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Practical impact of the newest achievements in assisted reproductive technologies (ART)

Edited by
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Practical impact of the newest achievements in assisted reproductive technologies (ART)

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Editorial: Practical impact of the newest achievements in assisted reproductive technologies (ART)

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Editorial on the Research Topic
Practical impact of the newest achievements in assisted reproductive technologies (ART)

In the four decades of ART history, several landmarks have stood out as turning points that have endurably changed the medical management of infertility. It is certain that these breakthroughs have recently had an impact on our daily practice of infertility management, so they deserve sets of reviews and updates that are gathered here. In this Research Topic, authors have provided a series of articles that provide a comprehensive update to the effectiveness of ARTs, their application, and concerns that might arise with their use.

Infertility-associated complications and risk factors

The increased resort to assisted reproductive technologies (ART) has boosted the research in this field. Increasing maternal age is one of the most common features of women seeking a pregnancy through ART. Two original studies evaluated maternal and neonatal risks associated to ART and increased maternal age, whilst a third one the impact of general and life-style risk factors on fertility.

In the first study, Tai et al. compared these outcomes in spontaneous pregnancies with pregnancies achieved after ART. Authors showed that several of the most common maternal and neonatal complications, were associated to ART. These included gestational diabetes, preeclampsia, moderate or severe anemia, liver and thyroid-related pathologies, preterm birth, placenta praevia, postpartum hemorrhage, cesarean section as well as fetal growth restriction, stillbirth and fetal malformations. According to the authors, the increased risk was only partially explained by the higher incidence of multiple pregnancies in the ART group.

Bouzaglou et al. conducted a study for assessing the most common maternal and neonatal outcomes in women aged between 40 and 45 years old compared to women aged between 25 and 35 years old. As expected, later maternal age resulted to be an independent risk factor for some major obstetrical complications, including cesarean section, preterm delivery, pre-eclampsia, gestational diabetes, and intrauterine fetal demise. Authors concluded that later age patients consulting for ART should be informed of these risks.

A further manuscript focused on semen quality, as a major constraint for ART, such quality having recently and progressively degraded according to several life-style changes. Thus, developing an algorithm able to predict semen quality would be worthy of interest. Zhou et al. developed a machine learning based model toward this aim. Besides smoking status and age, several further life-style and general factors were included in this algorithm.

Ovarian stimulation and oocytes retrieval

One of the primary adjuncts of ART, ovarian stimulation (OS), still stands today as the most effective measure ever implemented for improving ART outcomes. Yet, the equation that commands the control of the number of oocytes retrieved and embryo transferred has been drastically upended by access to embryo-vitrification.

Pirtea et al. go through and discuss the most relevant issues and advances in ART in this domain. Authors highlight in their review how the access to more flexible and secured treatments, including the use of antagonist protocols and GnRH-a for triggering oocyte maturation have reduced the risk of ovarian hyperstimulation syndrome (OHSS). On the other hand, the higher implantation rate achieved with ART has prompted physicians to switch to considering a single embryo transfer in order to reduce the incidence of multiple pregnancies.

Two further original articles of this series face two still unresolved problems of ART, the prediction of ovarian response and the prediction of developing a severe OHSS after OS.

In order to search for new parameters for predicting ovarian response to OS, Poulain et al. evaluated in their retrospective study for this scope the usefulness of calculating the follicle-to-oocyte (FOI) ratio defined by the number of retrieved oocytes divided by the antral follicular count (AFC) score (Oc/AFC). The ratio of the total number of mature oocytes (MII) and AFC (MII/AFC) was also evaluated. Results showed that an increase of all these three ratios was associated with the occurrence of live birth and implantation rate after first and cumulative embryo transfer.

Li et al. aimed in their study to search for new reliable markers of OHSS in peripheral blood samples of women undergoing a controlled ovarian hyperstimulation (COH) for oocyte retrieval. Four biomarkers of coagulation and fibrinolysis were evaluated concerning their performance in diagnostic and classification of OHSS. Results showed excellent diagnostic value, above 90%, for diagnosis of OHSS. Results were encouraging also concerning the potential to correctly classify the OHSS in mild-moderate vs. severe. According to specific cutoff values proposed, authors were able to exclude or even predict a high risk of developing severe OHSS in patients with mild–moderate OHSS. Results are noteworthy, as diagnosing and predicting the risk of developing a severe OHSS in women undergoing COH represents one of the major topics of research still in progress, with relevant medical impact in infertility treatment (Li et al.).

Tubal factor

Tubal infertility is a further common reason for female infertility. Several non-invasive diagnostic tools have been proposed in the past in order to avert anesthesiological and surgical risks of laparoscopy, which currently remain the diagnostic gold standard.

Tan et al. evaluated in their retrospective study the predictive value of hysterosalpingograpy (HSG) compared to laparoscopy for the diagnosis of tubal factor in infertility. According to the authors HSG has a good predictive value and can be adopted as a cost-effective first-line diagnostic procedure. However, authors underline that laparoscopy should still be considered as a complementary procedure in the diagnostic work-up of infertility.

Endometrial receptivity

The understanding of endometrial receptivity and its hormonal control has been recently updated through the study of euploid-embryo-transfers and its relationship with inflammation.

Bao et al. evaluated the potential association between ABO blood groups and immune response linked to infertility. Indeed, even though ABO blood group antigens seems to be linked to the development of several human diseases, there is still lack of consent in the current literature concerning blood group and ART outcomes. The authors found in their retrospective cohort study a positive influence of parental blood group AB compared to other blood group types on pregnancy and live birth rate in patients undergoing *in vitro* fertilization (IVF)/ intracytoplasmic sperm injection (ICSI) procedures. Considering this risk factor when counseling infertile couples seems to be noteworthy.

Chromosomal polymorphism (CPM) is considered to be a further aspect to be addressed during preimplantation genetic analysis (PGA) and ART procedures, particularly in case of unexplained recurrent pregnancy loss (uRPL). It remains,

however, unclear the level at which the fertility process could be impacted. Cao et al. found that male CPM have a higher aneuploidy rate and a lower blastocyst rate compared to female CPM. Interestingly, the transfer of an euploid blastocyst with male CPM achieved the same success rate in terms of pregnancy outcomes compared to blastocysts with a female CPM. Thus, according to these results, evaluating male CPM during PGA could improve ART outcomes, at least in uRPL.

Uterine factor

Uterine factor infertility (UFI) remains an unmet therapy—the last frontier of infertility—for which a new option, the uterine transplantation, has recently emerged. Several aspects of UFI have been reviewed, and look at cases suffering from an absence of the uterus, congenital or acquired, and/or definitive non-functionality of the uterus (Asherman Syndrome).

Favre-Inhofer et al. discuss in their review the importance of involving animal models in uterine transplantation, including the advantages and limits of each model currently in use. This overview highlights how the use of large animal models seems to be a mandatory preliminary step in order to reduce the morbidity and improve the success rate in human uterine transplantation.

Also, Sebbag et al. evaluated in their retrospective study the prevalence of intrauterine post-surgical adhesions by means of an early second-look hysteroscopy after an operative hysteroscopy. Overall, intrauterine adhesions were found in 18.7% of the women evaluated, being more frequent in women who underwent hysteroscopic lysis of adhesions (26.9%) and myomectomy (20.5), but also after polypectomy (10.9%). Data presented show a high prevalence of intrauterine adhesions and seem to support a policy of an early second-look hysteroscopy after an operative hysteroscopy.

Fertility preservation

Finally, another major topic to be considered in ART research, fertility preservation, has been tackled. Patients at risk for premature ovarian insufficiency, both in the case of chromosomal anomalies, and in the case of toxic or immunologic impairment, need, indeed, to be properly counseled concerning current options for preserving future fertility.

Ye et al. review in their manuscript the latest knowledge concerning strategies for fertility preservation in Turner syndrome. As premature ovarian failure is a common occurrence in Turner syndrome, cryopreservation of oocytes, ovarian tissues, and eventually of embryos should be discussed as early as possible. In case the ovarian reserve is already lost, oocyte or embryo donation, gestational surrogacy, and adoption should be considered.

Chen et al. go a step further and review current knowledge on fertility preservation in case of cancer, including special situations like prepuberal status and immediate need for chemotherapy. They go through current management options in case of cancer, like ovarian tissue cryopreservation and auto-transplantation with the linked risk of also retransplanting malignant cells. Interestingly, authors also present the latest research updates in modeling an artificial ovary, combining tissue engineering and stem cell research.

Condensation

Reproductive medicine has made constant new achievements with the development of new therapeutic options. We can undoubtedly state that today most infertility problems can be effectively treated by Assisted Reproductive Technique (ART); a fact that has remarkably reduced the number of couples who remain childless. In this Research Topic, we collected articles that provide a comprehensive update to the effectiveness of ARTs and their application to different types of infertility issues.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Early Second-Look Hysteroscopy: Prevention and Treatment of Intrauterine Post-surgical Adhesions

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Introduction: Intra-uterine adhesion (IUA) is one of the main causes of secondary infertility. The aim of this study was to evaluate the prevalence of IUA developing in women undergoing hysteroscopic resection for submucous myomas, polyps, and intrauterine synechiae and test the efficacy of second look hysteroscopy for diagnosing and treating post-surgical adhesions.

Materials and Methods: We retrospectively collected data from reproductive age women who had a second look office hysteroscopy following hysteroscopic resection for myoma, polyp, or IUA at Foch hospital (Suresnes, France) between 2009 and 2017.

Results: Six hundred and twenty two reproductive-age women underwent hysteroscopic resection for myoma, polyp, and/or IUA. Among them, 155 women had a second look hysteroscopy. In this group, 29/155 (18.7%) had IUA formation: 17/83 (20.5%) women who underwent hysteroscopic myomectomy, 5/46 (10.9%) women who underwent hysteroscopic polypectomy, and 7/26 (26.9%) women who underwent hysteroscopic lysis of adhesions. These IUA have been lysed by the office hysteroscopy procedure in 16/29 (55.2%) patients: 11/17 (64.7%), 2/5 (40%), and 3/7 (42.9%) in women who underwent hysteroscopic myomectomy, polypectomy and lysis of adhesion, respectively.

Conclusion: IUA is a common complication of hysteroscopic surgery. Second look office hysteroscopy is an easy and effective procedure for diagnosing and removing newly formed IUA. It should be recommended for all women undergoing hysteroscopic resection for myomas, polyps, or IUA.

Keywords: second look hysteroscopy, office hysteroscopy, adhesion prevention, intra uterine synechiae, intra-uterine adhesions, hysteroscopic resection

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INTRODUCTION

Intra-uterine adhesion (IUA) is an inflammatory reaction linking the opposed uterine walls. The resulting obliteration of the uterine cavity can be partial or total (Asherman's syndrome) (1). It most commonly occurs after traumas to the *basalis layer* of the endometrium most frequently, after curettage, cesarean sections, post-partum hemorrhage, abdominal myomectomy, and/or hysteroscopic resections of myoma, polyp, or uterine septum (2, 3). More rarely, it may also occur secondarily to infection (such as genital tuberculosis, endometritis).

IUAs can be responsible of symptoms including, chronic abdominal pain, menstrual abnormalities, and/or cause obstetric consequences like miscarriages, premature rupture of membranes (PROM), premature delivery, or placenta accreta (2). Furthermore, IUA is one of the main cause of secondary infertility with an incidence of 1.7–7% seen when hysteroscopies are performed in infertile women (4). We also know that hysteroscopic lysis of adhesion improves birth rate (32–46%) (5–10). Currently, hysteroscopy is the gold standard for the diagnosis and treating IUA (11). IUA can be classified in three stages, according to the American Fertility Society (AFS): mild (grade I), moderate (grade II), and severe (grade III) (12).

The purpose of the present study is to analyze prevalence of IUA development in women undergoing hysteroscopic resection for submucous myomas, polyps, and intrauterine synechiae and evaluate the efficiency of a second look hysteroscopy in order to diagnose and treat post-surgical adhesions.

MATERIALS AND METHODS

All patients undergoing surgery at our institution provided an informed consent authorizing the further analyses of their anonymized data, so Institutional Review Board approval was not necessary for this retrospective study.

In this retrospective cohort study, 622 reproductive-age women, between 18 and 45 years of age, underwent hysteroscopic resection for myoma, polyp, or IUA at our institution, Foch hospital (Suresnes, France) between January 2009 and March 2017. Among them, 155 patients had a second look office hysteroscopy following hysteroscopic surgery, 83 after myomectomy, 46 after polypectomy, and 26 after hysteroscopic lysis of adhesion for IUA. In the majority of cases (92/155), office hysteroscopy was performed as a second look procedure after an average time of 10.5 weeks. In the remaining cases (63/155) the post-surgical hysteroscopy was performed for other indications [infertility investigation, pre-IVF, atypical endometrial hyperplasia (AEH), dysfunctional uterine bleeding] with an average time interval of 47.4 weeks after the original surgery. A second look hysteroscopy was performed nearly systematically following hysteroscopic surgery starting from 2015. Data were collected from the operative and clinic records.

Technique

The original hysteroscopic surgery was performed under general anesthesia during the first part of the menstrual cycle. A 30° forward-oblique resectoscope with an outer diameter of 9 mm was introduced and the resection was performed with monopolar (resectoscope Karl Storz, Tuttlingen, Germany) or bipolar energy (VersaPoint® Gynecare, Somerville, NJ, USA) after prudent dilatation of the cervix by Hegar dilators. Physiologic saline (for bipolar resection) or glycine (for monopolar resection) was used to distend the uterine cavity. Antiadhesive gel (Hyalobarrier®) was freely used by surgeons. No antibiotics, hormonal therapy or IUD was used to prevent IUA.

The second look office hysteroscopy was performed without anesthesia. A 30° forward-oblique rectoscope (Bettocchi hysteroscope, Karl Storz®, Germany) with an outer diameter of 3 mm was used, without previous cervical dilatation. Physiologic saline or CO_2 was used to distend the uterine cavity. The procedure allowed the diagnosis of post-surgical adhesions and also to perform adhesiolysis of newly formed mild IUA with the tip of the office hysteroscope. If recurrent IUA were too thick and had to be surgically excised repeat surgical hysteroscopy was planned.

RESULTS

The median age of women at the time of the surgery was 36.6 years. Mean gestation and parity were 1.3 and 0.4, respectively. In total, 81 (52.3%) patients had a past surgical history including, curettage for incomplete or elective abortion (14.8%), operative hysteroscopy for myoma (14.2%), polyp (5.2%) or IUA (1.3%), abdominal (7.1%) or laparoscopic (0.6%) myomectomy, and cesarean (5.2%). Ninety-five out of 155 patients (61.3%) suffered from infertility at the time of original surgery: 48 primary infertility (31%) and 47 secondary infertility (30.3%) (**Table 1**).

Among the 155 patients, 7 had complications during the original surgery. Five patients had hydro-electrolytic disorders mainly hyponatremia due to the use of glycine, which resolved spontaneously and did not require intensive care. One patient experienced a post-operative hemorrhage with hypotension, tachychardia, and hemoglobin value of 6 g/dL, which motivated a laparoscopic exploration during which we found no hemoperitoneum but a vaginal wound caused by cervical dilatation, which was sutured. Another patient developed a per-operative bronchospasm and hypotension, which required intensive care treatment with orotracheal intubation, use of adrenaline and ephedrine. The bronchospasm resulted from an allergy to anesthetics products, which led to stop the surgical

TABLE 1 | Demographic characteristics.

Characteristics	Patients ($n = 155$)
Age (years)	36.6 ± 4.8
Weight (kg)	68.8 ± 16.2
Height (m)	1.65 ± 0.065
Body mass index (kg/m^2)	25.4 ± 5.8
Gestity	1.3 ± 1.8
Parity	0.4 ± 0.9
Tobacco use (%)	4 (2.58%)
Surgical history (%)	81 (52.3%)
Curettage (%)	23 (14.8%)
Hysteroscopic polypectomy (%)	8 (5.2%)
Hysteroscopic myomectomy (%)	22 (14.2%)
Hysteroscopic adhesiolysis (%)	2 (1.3%)
Laparoscopic myomectomy (%)	1 (0.6%)
Abdominal myomectomy (%)	11 (7.1%)
Cesarean (%)	8 (5.2%)
Infertility (%)	95 (61.3%)
Primary infertility (%)	48 (31%)
Secondary infertility (%)	47 (30.3%)

intervention. No cases of uterine perforation occurred. No complications happened during the second look hysteroscopy.

The postoperative office hysteroscopy revealed that 29 (18.7%) patients had IUA formations: 17/83 (20.5%) women who underwent hysteroscopic myomectomy, 5/46 (10.9%) women who underwent hysteroscopic polypectomy, and 7/26 (26.9%) women who underwent hysteroscopic adhesiolysis. These IUA have been lysed by the office hysteroscopy in 16/29 (55.2%) patients: 11/17 (64.7%), 2/5 (40%), and 3/7 (42.9%) women who underwent, respectively, hysteroscopic myomectomy, polypectomy, and adhesiolysis (**Table 2**).

TABLE 2 | Office hysteroscopy results.

Surgery indication	Patients	Hysteroscopic diagnostic of IUA	Hysteroscopic lysis of IUA
Myoma	83 (53.5%)	17/83 (20.5%)	11/17 (64.7%)
Polyp	46 (29.7%)	5/46 (10.9%)	2/5 (40%)
Synechiae	26 (16.8%)	7/26 (26.9%)	3/7 (42.9%)
Total	155	29/155 (18.7%)	16/29 (55.2%)

IUA, intra-uterine adhesion.

DISCUSSION

The current study shows that IUA is a common finding following hysteroscopic surgery, with an incidence of 18.7% found at second look hysteroscopy. While IUA occurred most commonly after hysteroscopic lysis of adhesion (26.9%) and myomectomy (20.5%), they were also seen after hysteroscopic polypectomy (10.9%). We found that in 55.2% of cases, IUA could be treated by second-look hysteroscopy. These results therefore validate the need for performing a second look diagnostic hysteroscopy following surgical hysteroscopies.

Few studies have focused on second look office hysteroscopy as a preventive measure of post-operative IUA and only a small number of patients were included.

Pabuccu et al. (13) randomized 71 women who underwent hysteroscopic lysis of adhesion into 2 groups. Thirty-six patients in group 1 had a second-look hysteroscopy 1 week following surgery (with further IUA lysis) and a third-look hysteroscopy 2 months later. Thirty-five patients in group 2 had a second-look hysteroscopy 2 months later. Both groups had an intrauterine device (IUD) inserted during the original hysteroscopic lysis of adhesion and received 2 months of estrogen and progestin therapy. The IUA formation rate was significantly lower in Group 1: no adhesion was detected in 33 (89.1%) of 36 patients in group 1 and 6 (17.1%) of 35 patients in group 2 ($p < 0.05$). Globally, these data show that early second-look hysteroscopy improves the ultimate success of surgery.

Robinson et al. (14) retrospectively evaluated 24 patients treated with primary hysteroscopic lysis of adhesion followed by hormone therapy and serial flexible office hysteroscopy. They found that 92% (22/24) of patients had an overall improvement in the stage of their Asherman's syndrome.

Yang et al. (15) reported data on 153 women who had a hysteroscopic myomectomy for single or multiple (apposing or not) myoma. They were divided into 4 groups with different IUA prevention strategies. Diagnostic office hysteroscopy was done 1–3 months after surgery and revealed that postoperative adhesions are common in women who had apposing myomas (despite IUD) but were not found in any of the women ($n = 7$) undergoing office hysteroscopic early lysis.

The optimal interval for realizing the second look hysteroscopy has not been established yet but it is believed that early dissection during second look hysteroscopy has a positive outcome on the ultimate risk of developing new synechiae. Some authors recommend very early hysteroscopy (13, 15), however there is no solid evidence for such claim. One of the limitations of this study is the various time interval between the operative and the office hysteroscopy, but most of them were performed early after the surgery as a second look procedure.

According to Shokeir et al. (16) IUAs formed immediately after the surgery are histologically different from those appearing a longer time after the operation. Early occurring IUAs are mainly composed of grade I vs. grade II/III. Indeed, early office hysteroscopy allows the lysis of newly formed adhesions, which are thin and filmy, whereas delayed adhesions are thick and fibrous and need a surgical lysis of adhesion (14). In this study we unfortunately did not have data about adhesion types.

Office hysteroscopy may be a way of verifying the effectiveness of preventive anti-adhesives. Various measures of preventing adhesions were studied. Anti-adhesive gels, such as hyaluronic acid gel (Hyalobarrier®) are the most widely used and have a significant clinical effect on IUA prevention (17–19). However, their effect on further pregnancy rate is unknown. These results must be confirmed by prospective randomized trials before we can recommend their general use. The role of intrauterine device (IUD), hormonal and antibiotic therapy are difficult to evaluate because generally, these have been used in association with other prevention strategies (4). Only one low-power randomized trial studied the benefit of the intrauterine device (IUD) with or without estrogen treatment to prevent IUA after operative hysteroscopy but no significant result was found (20). Neither technique can be recommended for routine use (11). Finally, bipolar resection seems to be associated with a lower IUA recurrence rate compared to monopolar resection, but randomized prospective studies are still needed to confirm this result (21).

Regarding reproductive outcomes, no study has demonstrated its effectiveness in improving spontaneous fertility but it seems that control hysteroscopy may improve pregnancy rates: 47 vs. 30% (13).

The main limitation of this study is its retrospective nature. Followed by the small number of patients who underwent second look hysteroscopy. Unfortunately due to the retrospective design of the study, we were not able to collect subsequent fertility data, which could have made the study more interesting. Therefore high-quality randomized studies are still needed to strongly recommend systematic office hysteroscopy after hysteroscopic resection for submucosal myomas, polyps, or synechiae.

Second look office hysteroscopy is shown to be a simple, safe and useful procedure for investigating intrauterine lesions. It allows identification of post-surgical adhesions and early treatment before they become thick and a new intervention is needed.

CONCLUSION

In conclusion, second look office hysteroscopy is an easy and effective procedure for diagnosing and removing newly formed IUA. Second look hysteroscopy could prevent the development of moderate or IUA and therefore improve reproductive outcome. Routine early second-look hysteroscopy should be recommended for all women undergoing hysteroscopic resection for myomas, polyps, or synechiae within 6 weeks after the surgery.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All patients undergoing surgery at our institution provided an informed consent authorizing the further analysis of their anonymized data, so Institutional Review Board approval and written consent was not necessary for this retrospective study in accordance with the local legislation and institutional requirements. According to the French Public Health Code (article L1123-7-25th May 2018), only research which involves directly the patient participation requires an approval from an IRB/IEC. In the case of our research, only data collection from medical records has been performed. This kind of research does not involve directly the patient participation, does not requires ethical review or written consent.

AUTHOR CONTRIBUTIONS

LS and ME contributed to the protocol and project development, data collection and management, data analysis, and manuscript writing and editing. SF, IN, AR, MC, and PP contributed to the protocol and project development. DZ and J-MA contributed to the manuscript writing and editing.

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REVIEW
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New Twists in Ovarian Stimulation and Their Practical Implications

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Ovarian stimulation (OS) has for objective to induce multiple ovulation in order to yield a multiple oocyte harvest and offer multiple embryos available for transfer thereby increasing the efficacy of ART. Originally, the primary risk associated with OS was the occurrence of frank ovarian hyperstimulation syndrome (OHSS), a possibly dreadful—sometime fatal—complication of ART. These fears limited the number of oocytes aimed for during OS in order to curb the risk of OHSS. On the contrary, the meager implantation rates of the early days of ART led to easily transfer multiple embryos in order to achieve acceptable pregnancy rates. Today the perspectives have changed. The advent of antagonist-based OS protocol and the possibility to trigger the ultimate phase of oocyte maturation with GnRH-a has allowed to reduce the risk of OHHS. Conversely, the markedly increased implantation rates of today's ART makes multiple pregnancy a worry that has come in the limelight worldwide, pushing the practice of single embryo transfer (SET).

Keywords: ovarian stimulation, gonadotropin, ovarian hyperstimulation syndrome (OHSS), GnRH antagonist, dual ovarian stimulation

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INTRODUCTION

While the first successful ART pregnancy has emanated from an oocyte retrieval conducted in the natural cycle, ovarian stimulation (OS) very soon became associated to ART. Indeed, OS allows to increase the number of oocytes available and in turn the cohort of available embryos to choose from at the time of transfer. Today four decades into the history of ART, OS stands as the single most effective measure ever enacted to increase the yields—implantation and pregnancy rates—of ART. Yet the original equation that determined the constraints on the number of follicles stimulated and embryos transferred has recently changed. Indeed, the core of this brief review article focusses on the fact that a more liberal attitude generally prevails today regarding the number follicles stimulated, while efforts have focused on preferring single embryo transfers (SET).

OOCYTE QUALITY AND QUANTITY

In the yesteryears of pre-ART times, the progressive decrease in fertility and increase in miscarriage rates observed as women grow older was for the larger part attributed to an aging process affecting the uterus (1). The dogma proffered that the aging uterus could not allow a proper attachment of the developing embryo. ART data have upended these views however. We now know through four decades of ART experience that the age-related decrease in implantation rates and increased in miscarriage is principally, if not solely, due to a decrease in oocyte quality. Indeed, the long recognized deterioration of the reproductive potential seen when women become older—lower pregnancy rates and increased miscarriage rates—is only seen in autologous ART. Conversely,

implantation and miscarriage rates remain constant in donor-egg ART until the age of 50 years and beyond (2). This therefore clearly indicates that the decreased implantation rates and increased miscarriage rates seen in ART in older women only reflects a decrease in oocyte quality, not a uterine phenomenon (3). In aging donor-egg ART recipients, implantation, and miscarriage rates are similar to those of younger women, being solely dependent upon the age of the oocyte donor (3).

There is an erroneous belief that oocyte quality and quantity are inherently linked. Contributing to the concept that the declines in oocyte quality and quantity are linked is the fact that age induces a parallel downturn of both parameters. In an extensive study, Faddy et al. reported that the decline in total primordial follicles abruptly increases by the age of 37 years (4), as illustrated in **Figure 1**. The assertion that the decline in oocyte quality and quantity are fundamentally link is erroneous however. The appearance of a causal link is in fact solely the result of the effects of a confounding factor—age—which affects both oocyte quality and quantity (5). This dual effect of age explains that in older women, the so-called poor ovarian responses—insufficient oocyte yield—are commonly associated with oocytes that are also of poorer quality and lesser chances of providing a pregnancy (6). Yet as discussed below, when the decrease in oocytes is either constitutional or due to an age-independent factor, there is not necessarily a decrease in oocyte quality (7).

When oocyte quantity is impaired due to an age-independent factor such as for example endometriosis or past surgery for endometriosis, evidences indicate that oocyte quality is not decreased (7). In a retrospective cohort analysis, we showed that endometriosis is not associated with decreased oocyte quality. This was evidenced by the fact that we observed similar aneuploidy rates in endometriosis and age-matched controls (8). The fact that ART results are not necessarily reduced in endometriosis despite poorer responses to OS supports the concept that age-independent decreases in oocyte quantity are not associated with a parallel decrease in oocyte quality.

Similarly, certain women have smaller number of antral follicles despite being young. These women albeit young have poor ovarian reserve parameters such as notably, AMH levels and antral follicle count (AFC). In a series of women who had conceived and ultimately delivered a live child within 15 months of discontinuing the OC pill, we observed no correlation between AMH levels and effective time to pregnancy (9). Hence, poor ovarian reserve parameters are not *per se* associated with a decrease in natural fecundity. Likewise, a significant proportion of fertile egg donors showed a poor ovarian response to OS within a mean interval of 2 years after delivering their last child (10).

Practically therefore, a small oocyte crop obtained in women whose limited number of oocytes is not due to age warrants a different approach when managing ART than when poor responses are due to age. In the former cases, a small number of oocytes is worth harvesting because pregnancy chances are preserved contrary to what is seen in women whose poor response is due to age (6). In the latter case, a poor response—$\leq$3 oocytes expected—due to an age-related decline in ovarian response probably questions the worthiness of proceeding to oocyte retrieval.

There have been proponents of reducing the amount of gonadotropin used in OS in a strategy called mild stimulation (mOS). The purpose pursued was to reduce the number of oocytes retrieved and the possible complications of OS such as notably, OHSS while retaining a fair number of good quality oocytes. Today we know that the risk of OHSS is not due to the number of follicles responding to OS, but rather to an effect of hCG—administered for triggering ovulation or produced by the developing conceptus—on ovarian follicles. These new perspectives on OHSS certainly reduced the interest for mOS as a primary mean of curbing the risk of OHSS. Indeed, the primary defense for reducing OHSS is to avoid using hCG for triggering ovulation and proceed to a freeze all approach, which will prevent an effect trophoblastic hCG on ovarian follicles and late-onset OHSS. As this strategy

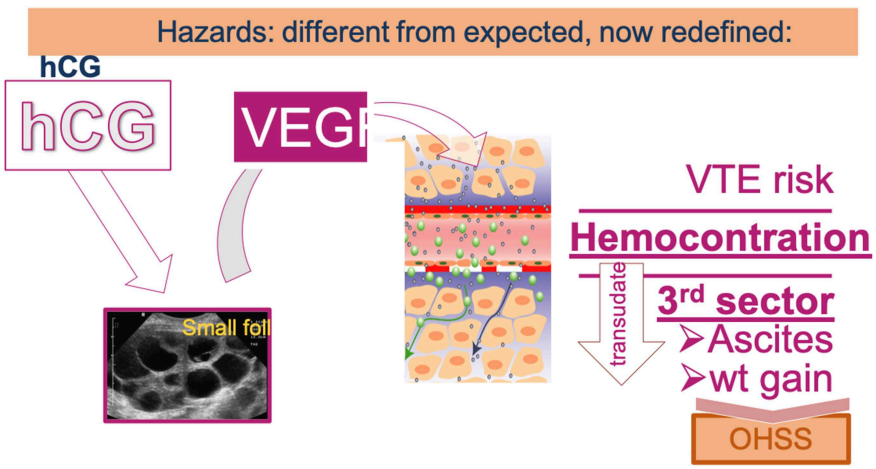

FIGURE 1 | Paralell decline in oocyte quality and quantity—primordial follicle count—as a function of age (adapted from Faddy et al. with permission).

has practically reduced the risk of OHSS, the justification for mild stimulation is therefore reduced. Moreover, the advent of pregestational testing for aneuploidy (PGTA) and its rapid expansion in our everyday ART practice favors increasing the number of embryos available for testing. Consonant with this view, Rodriguez-Purata and Martinez (11) states that "the aim of ovarian stimulation has shifted from obtaining embryos available for transfer to yielding the maximum embryos available for biopsy to increase the odds of achieving one euploid embryo available for transfer" (11). Nevertheless, mild OS still has its protagonists (12). These authors predict "widespread acceptance of mild IVF, by both patients and IVF providers, and make IVF more accessible to women and couples worldwide," as mOS reduces the cost and side-effects associated with ART (12). In an editorial comment in Fertility and Sterility, Paulson R expresses similar foresights saying, "As the treatment of infertility becomes more personalized, it is likely that standard, heavy-handed stimulation protocols will give way to simpler strategies" (13). Conversely however, numerous voices also rose to indicate that mOS offers a lesser ART yield than regular OS protocols. Based on a systematic review of the literature Orvieto et al. concludes that "an objective review of the literature does not support the routine utilization of mOS in ART (14). Likewise, in a case-control study, Siristatidis et al. conclude that mOS regimens using either CC or letrozole do not seem to constitute an equally effective method as compared to the conventional OS protocols in good prognosis subfertile women (15). Further studies are therefore needed to definitively assess the role of mOS in ART. In the meantime, careful assessment of the personal history and objectives—i.e., PGTA or not—should guide clinicians in their choice of OS protocol.

OVARIAN STIMULATION DOES NOT HAMPER EMBRYO QUALITY

Originally, there were concerns that excessive ovarian responses led to crops of oocytes of poorer quality (16). This diehard belief is however not supported by recent data. In one set of evidence oocyte quality was assessed by the incidence of first trimester miscarriages (17). The authors of this work based on UK registry data of 124,351 ART cases observed an inverse correlation between the number of oocytes retrieved and the risk of miscarriage (17). Of course, women who produced more oocytes tended to be younger and are therefore less prone to miscarry. Data of this study were then sub-analyzed by age groups. While as it could be expected the miscarriage risk increased with age, in each age group there was no negative impact of the number of oocytes retrieved, but rather a trend toward lesser risks of miscarriages in women who got more oocytes (17).

Concordant with the above results there was no correlation between the amounts of FSH/hMG used during OS or the number of oocytes retrieved and the euploidy risk in each age group (18). Likewise, implantation rates of euploid embryos—constant in all age groups—were not affected by the amounts of gonadotropin used during OS (18). Furthermore, the impression that there was an optimal number of oocytes retrieved—15—beyond which any further increase failed to further enhance outcome is challenged if cumulative pregnancy rates are considered. In a recent set of data, Drakopoulos et al. indicated that cumulative pregnancy rates continued to increase with oocyte retrievals that exceeded 15 oocytes (19). These authors however reported an increased risk of OHSS in the high responders. Probably however having reverted to GnRH-a only for triggering the final stages of oocyte maturation and deferred embryo transfer should have been more liberal in these cases.

These data put together with the fact that OHSS can effectively be prevented have drastically changed the terms of the OS equation. As discussed below, the current trend is to push toward harvesting more oocytes. Conversely however, the higher implantation and pregnancy rates of modern-day ART make us strive for the need of single embryo transfers (SET) whenever possible (20).

DUAL OR "DUPLEX" OVARIAN STIMULATION

From the onset of ART, OS was timed at the onset of the follicular phase for two primary reasons: First, to ensure that fresh embryo transfers took place during a receptive period; Second, there was an unproven belief that OS protocols had to act on antral follicles present in the early follicular phase for the fear that other hormonal environment—i.e., progesterone elevation—might negatively affect the quality of the harvested oocytes. Two factors have modified the terms of this fundamental principle that reigned over yesteryears' dogmas regarding OS in ART.

1. The advent of embryo vitrification replacing cryopreservation by the slow freezing approach freed ART from the need of transferring embryo in the luteal phase of OS without fearing a decrease in outcome. Indeed, freeze-all and deferred-embryo-transfers provide either improved or equal results as fresh transfer—based on the patient population—but never inferior results (21, 22). Hence, by freeing from the need of luteal phase transfer, OS could theoretically be initiated at times—including during the luteal phase—different than the early follicular phase (23). Reporting a meta-analysis and systematic review, Boots et al. report that luteal phase OS is equally effective as follicular phase OS even if there is a slight trend for longer stimulations and a higher dose of total gonadotropin (24).
2. The need of fertility preservation in women scheduled to initiate chemotherapy has led to initiate OS at times other than the early follicular phase—i.e., random start OS—due to the time constraints that exist in oncology. These approaches did not decrease OS efficacy (25). Further, a group in Shanghai demonstrated that luteal phase-initiated OS yielded comparable cumulative ART results—implantation and pregnancy rates—as follicular phase-started OS (23).

Furthermore, several groups have reported that two consecutive OS protocols could be initiated in the follicular phase and the

subsequent luteal phase. In the first report of this strategy—the Shanghai protocol—the authors obtained more oocytes from the luteal-phase OS. They therefore postulated that the first OS—follicular phase—exerted a priming effect on the ovarian response (26). There original protocol however used heftier OS doses during the luteal phase OS so that the priming effect of the follicular phase could not be confirmed however. Subsequently, we (27) as well as others (28) have shown that the follicular and luteal phase OS yielded a similar number of blastocysts, as illustrated in **Figure 2**. The latter authors (28) further showed that the embryo euploidy rates of the follicular and luteal phase were similar. The dual or "duplex" OS protocol is an effective way to increase the number of oocytes and embryo obtained over a relative short period of time. It has its place when the number of oocytes needs to be optimized over a short period of time, as for fertility preservation and in certain cases of poor responders.

Based on the duplex stimulation, the progestins (endogenous and exogenous) was administered to prevent the premature LH surge during ovarian stimulation (29) making way for new types

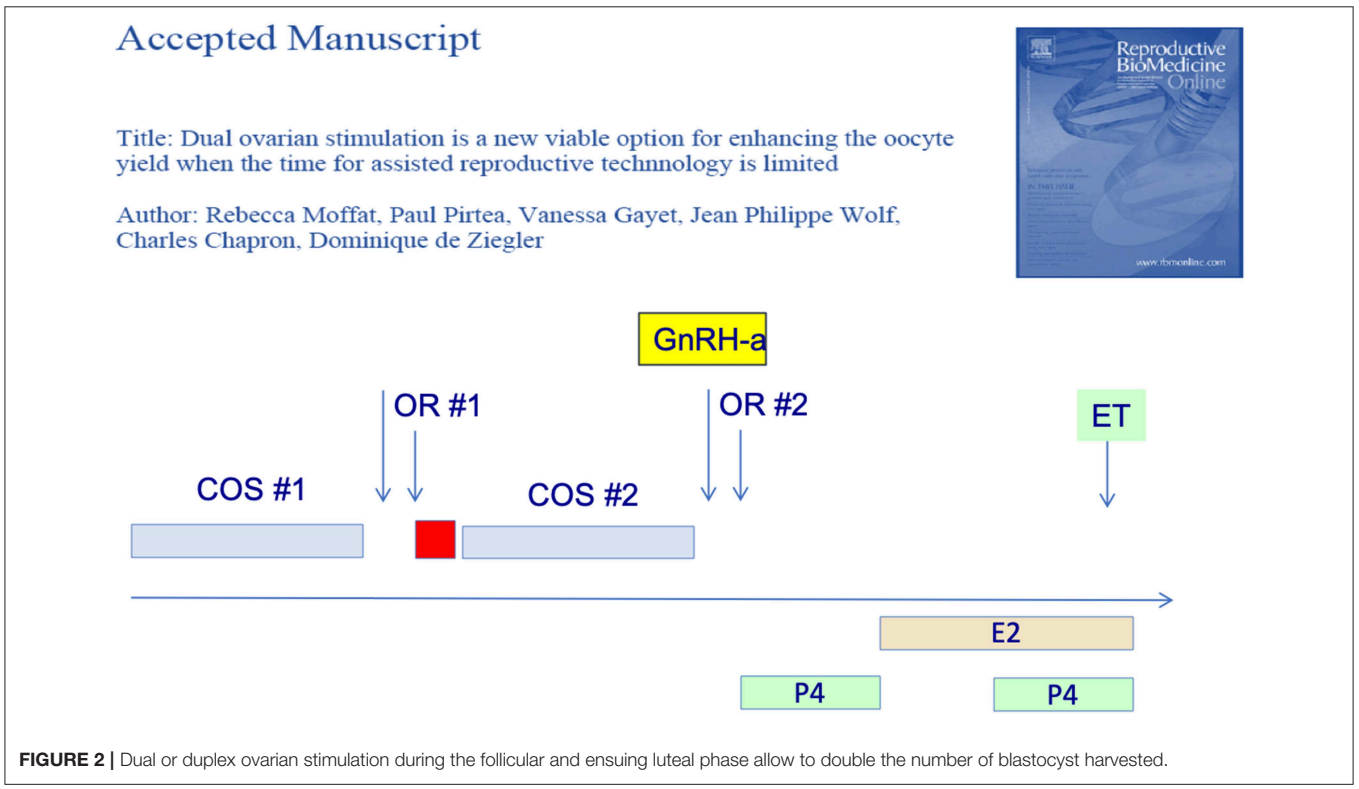

FIGURE 2 | Dual or duplex ovarian stimulation during the follicular and ensuing luteal phase allow to double the number of blastocyst harvested.

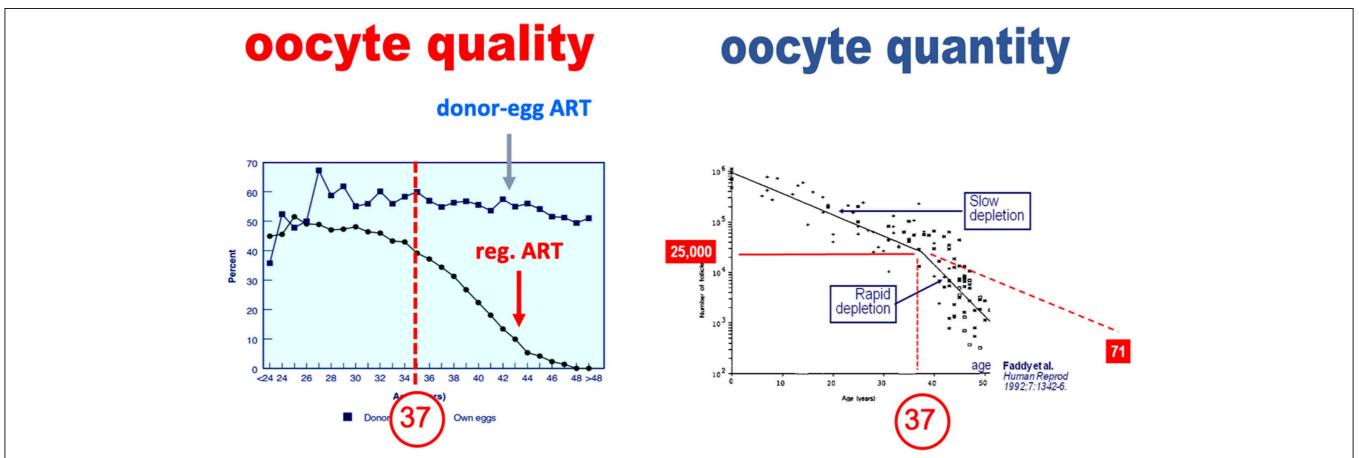

FIGURE 3 | Under the influence of hCG, the hyperstimulated ovary produces the vasoactive VEGF that modifies vascular permeability leading to an efflux of vascular fluid—forming oedema and ascitis—and also leading to hemoconcentration thereby increasing the risk of venothrombo embolism (VTE).

of stimulation protocols. Two ways of using progesterone have emerged, whether endogenous, as in luteal phase stimulation, or exogenous, as in the use of progesterone in the follicular phase i.e., progestin primed ovarian stimulation (PPOS). This type of protocol in combination with the so called freeze-all strategy is also useful for OHSS-free clinics (30, 31) and also can offer a break away from the standard methodology of stimulation-retrieval-transfer.

OHSS-FREE CLINICS

OHSS was a dreadful complication of OS which affected up to 2–3% of ART participants in its severe form requiring hospitalization and most often ascites aspiration, which often needed to be repeated. Furthermore, OHSS was associated with an increased risk of venous thrombo-embolism (VTE) and caused several fatal outcomes. The generalization of antagonist OS protocols and the possibly to revert to GnRH-a for triggering the last step of oocyte maturation has allowed to practically eradicate the risk of OHSS (32). There are indeed evidences that OHSS stems from an effect of hCG—used for triggering ovulation or produced by the conceptus—causing the production of vasoactive endothelial growth factor (VEGF) by the developing follicles, as illustrated in **Figure 3**. Hence, hCG-free OS protocols as notably using GnRH-a for triggering ovulation (33)—not attempting to rescue the luteal phase with small doses of hCG—and deferred embryo transfer reduce the risk of OHSS to practically zero.

Following the use of GnRH-a for triggering ovulation the luteal phase environment is so profoundly disturbed that fresh pregnancy rates are minimal when using the common modes of luteal phase support. This led the vast majority of practitioners to revert to freeze-all-and-deferred embryo transfer each GnRH-a is used for triggering ovulation. Others have opted for different protocol of minimal hCG supplement in order to rescue the luteal phase following GNRH-a triggering of final oocyte maturation (34). Strict adherence to freeze-all-and-deferred-embryo-transfer approach when GnRH-a is used offers the valuable advantage of having reduced the most dreadful complication of ART, OHSS.

CONCLUSION

Originally OS tended to be moderate for the fear of causing the dreadful—sometimes fatal—complication of ART, OHSS. Conversely, multiple embryo transfers were common to palliate the poor implantation rates that prevailed in the early days of ART and the relatively poor success of embryo freezing. Today the terms of the equation have changed. Energetic more productive and more flexible OS can be conducted without the fear of OHSS through antagonist protocols and the possibility of reverting to GnRH-a trigger for the final stage of oocyte maturation. On the contrary, the high implantation rates—notably of euploid embryos—command to most often if not always revert to SET in order eliminate the ART-increased risk in multiple pregnancy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Progress in Fertility Preservation Strategies in Turner Syndrome

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Growth retardation and gonadal dysgenesis are two of the most important clinical manifestations of Turner syndrome (TS). As premature ovarian failure generally occurs early in life in women with TS, these patients should be counseled and evaluated as early as possible for discussion of optimal and individualized fertility preservation strategies. Infertility seriously affects the quality of life of women with TS. For those who have ovarian reserve, the theoretical options for future fertility in TS patients include cryopreservation of oocytes, ovarian tissues, and embryos. For those who have already lost their ovarian reserve, oocyte or embryo donation, gestational surrogacy, and adoption are strategies that allow fulfillment of desire for parenting. This review describes the etiologies of infertility and reviews the fertility preservation strategies for women with TS.

Keywords: Turner syndrome, fertility preservation, cryopreservation of oocytes, cryopreservation of ovarian tissues, cryopreservation of embryos

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INTRODUCTION

Turner syndrome (TS) is one of the most common sex chromosome abnormality in women. The incidence rate of TS in female newborns is about 1/2,500 (1). The karyotypes of TS are 45,X monosomy accounting for about 50% of the cases, 45X/46XX mosaicism accounting for about 20–30%, and the rest is other X chromosome structural abnormalities (2). It is usually characterized by hypergonadotrophic hypogonadism due to gonadal dysgenesis leading to premature ovarian failure with subsequent infertility. About 80% of women with TS do not have spontaneous puberty, and the ovarian reserve in 90% of women with TS will be depleted before adulthood (3). Fertility preservation in women with TS needs to be offered in a timely fashion. In this paper we review available methods for the detection of ovarian reserve, as well as the different for fertility preservation in women with TS.

FERTILITY RESERVE AND OVARIAN RESERVE TESTING IN WOMEN WITH TS

Ovarian Reserve

Ovarian reserve refers to the number of primordial follicles contained in the human ovarian cortex. The formation of the ovarian reserve in females with normal chromosomal karyotype begins at the fetal stage. At that stage, 100–2,000 primitive germ cells enter the genital ridge and proliferate in large numbers, reaching the maximum amount of germ cells in the second trimester of pregnancy. After that, the number of germ cells gradually decreases, and no new germ cells are formed. Mamsen et al. (4) observed that the number of germ cells in 53 fetal ovaries at different stages

of pregnancy increased from an average of 7,200 at 7 weeks of gestation to 4,933,000 at 19 weeks of gestation, but about 85% of the germ cells were lost before birth and the number dropped to about 600,000 at birth. The number of oocytes decreases with age, regardless of whether a woman has an ovulatory cycle or not. By the age of 37, the number of eggs is ∼25,000, and the rate of reduction will be accelerated. By the age of 51, the number of remaining eggs will be <1,000 in the average woman (5).

Studies have shown that the ovarian development in fetuses with TS is normal before 12 weeks of gestation, but oocyte loss is accelerated in many of them after 18 weeks of gestation. In normal karyotype fetuses, oocyte division begins at 18 weeks of gestation, primordial follicles are observed at 20 weeks of gestation, and antral follicles are observed at 26 weeks of gestation. On the contrary, at the same gestational week, oocytes are present in the ovaries of 45,X fetuses, but no follicles are observed. Finally, the number of germ cells at birth is significantly less than that of females with normal karyotypes at the same developmental age (6, 7). Hook et al. (8) also observed that, in the early stage of a fetus with abnormal chromosomal karyotype, there were normal numbers of germ cells in the reproductive ridge. However, in the second trimester of pregnancy, the number of germ cells was significantly lower than that of the normal fetus, indicating that the rate of apoptosis of germ cells during development was higher in the fetus with abnormal chromosomal karyotype. Modi et al. (9) made a semi-quantitative analysis of the ovaries of 16 normal fetuses and 4 TS fetuses at 15–20 weeks of gestation, showing that TUNEL (terminal deoxynucleotidyl transferase-mediated deoxiuridinetriphosphate nick-end labeling) -positive cells were present in 3–7% of normal fetuses and 50–70% in fetuses with TS, also suggesting that the rate of apoptosis of reproductive cells in fetuses with TS was higher than that of normal fetuses. Therefore, it was speculated that the accelerated apoptosis of germ cells in women with TS in the intrauterine growth period may be the main mechanism of follicular depletion in this syndrome.

Chromosome Karyotype and Ovarian Reserve in TS

The chromosome karyotype of women with TS has been correlated with the size of ovarian primordial follicular pool and spontaneous puberty. Hankus et al. (10) compared the karyotypes of 110 patients aged 10.7 ± 4.0 years, and the results showed that in 48% patients with spontaneous puberty, most of them were non-45,X girls. Mamsen et al. (11) evaluated follicular density, morphology, and health in the ovarian cortex of 15 patients aged 5–22 years with TS, and found that 9 women with TS had follicles with chimeric karyotype. Bernard et al. (12) found that 27 patients among 480 women with TS (5.6%) had a total of 52 spontaneous pregnancies. The two predictive factors which correlated with the occurrence of spontaneous pregnancy were spontaneous menarche and mosaic karyotype. However, Mortensen et al. (13) reported that a small number of women with TS with karyotype 45,X can have spontaneous puberty and regular menstruation, and even spontaneous pregnancy. Therefore, it is difficult to accurately predict ovarian reserve and fertility potential in women with TS on the basis of chromosomal karyotype alone.

In addition, studies have shown that the chromosomal structure of women with TS has an impact on ovarian function. Hreinsson et al. (3) studied and carried out histological analysis on the ovaries of 9 women with TS, among which 8 had follicles. Their results showed that there were more follicles in the ovaries of patients with mosaicism and younger women with TS. Quilter et al. (14) reported that in a comparative genomic hybridization (aCGH) analysis of 42 patients with idiopathic POF (Premature ovarian failure), 15 were found to have copy number variations (CNV) on chromosome X and chromosome 7. Mercer et al. (15) conducted a more in-depth study on the fertility of 20 patients with long arm variations of the X chromosome, showing that loss of Xq26-Xq28 has the greatest impact on ovarian function. These studies suggest that abnormal X chromosome structure and even CNV on autosomes may be associated with the fertility of women with TS.

Evaluation for Potential Fertility in Women With TS

As the ovarian reserve capacity varies among women with TS, it is important to select these patients who can preserve their fertility as early as possible. Anti-Mullerian Hormone (AMH) is a reliable marker of follicular reserve in normal adults. Even though there was a study showing that AMH is suitable for use in young or adolescent women, there is few data available on AMH in childhood (16, 17). A retrospective study of 28 women with TS with different karyotypes was conducted by Lau et al. (18) to compare their development and menstruation, and to measure serum Follicle-Stimulating Hormone (FSH) levels in order to assess their fertility. The results showed that patients were more suitable for fertility preservation when they had spontaneous menarche, had at least one normal ovary confirmed by ultrasound, and their serum FSH levels were below 40 IU/L. Birgit et al. (19) reported on laparoscopic examination and ovarian biopsy in 57 girls with TS aged 8–19.5 years. Ovarian cortical tissues were obtained from 47 of these TS girls with non-streak ovaries for follicular analysis and cryopreservation. Luteinizing Hormone (LH) and FSH levels were measured in 30 of them, and AMH levels were measured in 43 of them. Follicles were detected in the ovaries of 26% of these women, and the number of follicles varied based on the levels of LH, FSH, and AMH, karyotype, spontaneous puberty and menarche.

Based on the above studies, it appears that women with TS may benefit by having an assessment of ovarian reserve at the age of 13 or 14, so as to consider their options for fertility preservation in the most timely manner. The diagnostic parameters are mainly serum AMH level, serum FSH level, karyotype analysis, spontaneous puberty development, ovarian volume detected by ultrasound examination and follicle count. Hagen et al. and Kelsey et al. research shows the serum AMH decreased earlier than FSH, inhibin B, and E2 in prepubertal girls (16, 20). Among them, serum FSH levels, ovarian volume measured by ultrasound examination and follicle count have strong periodicity, and are interdependable, whereas AMH is a

relatively stable hormone index (21). Recently, studies have also shown that there is no periodic fluctuation in serum AMH levels, and that it is better than FSH, E2, INH-B, and AFC in reflecting the downward trend of ovarian reserve function with age (22).

STRATEGIES FOR REPRODUCTIVE COUNSELING FOR WOMEN WITH TS

Oocyte Cryopreservation

Cryopreservation of oocytes is an established method that may be used for both mature and immature oocytes. The first live-born baby following this technique was born in 1986 (23). After decades of development, oocyte cryopreservation has made great progress, especially with the emergence of vitrification. The pregnancy rate and live birth rate of thawed oocytes transplanted after vitrified cryopreservation were similar to those of transplanted fresh oocytes (24).

As already mentioned, in most women with TS ovarian reserve may be depleted before or relatively soon after puberty. Moreover, women with TS may have oocytes with normal or abnormal karyotypes depending on their own karyotypes. Oocyte cryopreservation may provide a chance for selecting the normal karyotype oocytes resulting thus in an embryo with a normal karyotype. Therefore, offering cryopreservation with oocyte vitrification in patients in women with TS before ovarian reserve is a highly considerable option. Due to chromosomal abnormalities in women with TS, not all oocytes develop with a normal karyotype and resulting fertilized embryo's also carrying the chromosomal abnormality, with females developing TS and males being carriers of the condition. Fortunately, pre-implantation genetic screening (PGS) technology is mature and can be used to screen stored oocytes and embryos for chromosomal abnormalities. Given the technological advances in oocyte and embryo screening, it can be predicted that women with TS are expected to successfully conceive and produce healthy babies through these techniques.

Cryopreservation of Mature Oocytes

Mature oocytes mainly refer to the oocytes in the MII (meiosis II) stage. In natural cycles, only 1–2 oocytes at one time can reach full maturity at MII stage, and the rest will degenerate after atresia. Ovarian stimulation by exogenous gonadotropin administration to promote ovulation is the best approach to collect multiple mature oocytes in a relatively short period of time (25, 26). The follicular maturation is monitored by ultrasound, and then the oocyte is collected by ultrasound guided ovum retrieval. The collected oocytes are vitrified and cryopreserved, and the frozen oocytes will be thawed and fertilized at the appropriate future time (**Figure 1**). So far, this technique has been successfully used in a small number of women with TS (18, 25, 27, 28). Unfortunately, the number of mature oocytes retrieved in a single cycle is very small in women with TS. Therefore, the procedure often has to be repeated in order to finally obtain more than 10 mature oocytes. Then, it is difficult for a young patient (adulthood).

Cryopreservation and *in vitro* Maturation of Immature Oocytes

Immature oocytes refer to the oocytes at GV (germinal vesicle) or MI (meiosis I) stage. The collection of immature oocytes does not require gonadotropin stimulation. They may be directly harvested from removed ovarian tissue and their size usually ranges from 2 to 8 mm. *In vitro* maturation (IVM) of immature oocytes is also an assisted reproductive technology, which takes out the immature oocytes from the ovary, transfers them into appropriate culture medium simulating the follicular microenvironment *in vivo*, cultures the oocytes *in vitro* to mature stage, and then fertilization and pregnancy may occur through IVF-ET (29) (**Figure 1**). IVM can completely bypass ovulation promoting process, simplify IVF program and shorten treatment time, offering thus significant advantages.

Oocyte cryopreservation techniques use autologous oocytes and do not require sperm fertilization, thus avoiding religious and ethical issues. If a patient is unable to gestate because of pregnancy contraindications, as in a number of selected cases of TS, these techniques may also provide the possibility of retaining oocytes to achieve pregnancy via surrogacy. However, it should be noted that for women who cannot tolerate ovarian stimulation and for some TS girls who either do not have a partner or are at a very young, even prepubertal age, transvaginal oocyte retrieval may not be easily accepted by them or-in the latter case-by their parents, and may not be applicable to them. Transabdominal oocyte retrieval is not generally recommended, and in this case, a more appropriate alternative approach should be offered. Moreover, not all oocytes obtained are suitable for fertilization, and some oocytes may have abnormal karyotypes. Therefore, chromosome screening should be routinely carried out before embryo implantation in order to screen out aneuploidy embryos and to improve the outcome of pregnancy. However, this technology is currently being evaluated and has never been studied in women with TS.

Ovarian Tissue Cryopreservation and Ovarian Transplantation

Fertility preservation techniques for cryopreserved ovarian tissues are still in the experimental stage, but it has been reported that cryopreserved ovarian tissues after orthotopic transplantation can restore ovarian function and lead to spontaneous pregnancy. Donnez et al. (30) first reported in 2004 that a patient with premature ovarian failure caused by chemotherapy for Hodgkin's lymphoma had a successful pregnancy and delivered a baby after orthotopic transplantation of her cryopreserved ovarian tissue. According to relevant data, by 2017 the number of live births through this technology has exceeded 130 babies, with live birth rate constantly increasing. As the ovarian tissue is preserved in multiple pieces, the transplantation process can and may be repeated, providing each time a new opportunity to restore ovarian function. There have been reports that ovarian function can be restored for 4–5, or even 7 years. This technology can not only help preserve the fertility of patients, but also help restore endocrine function (31–35). However, so far, there have been no reports in women with

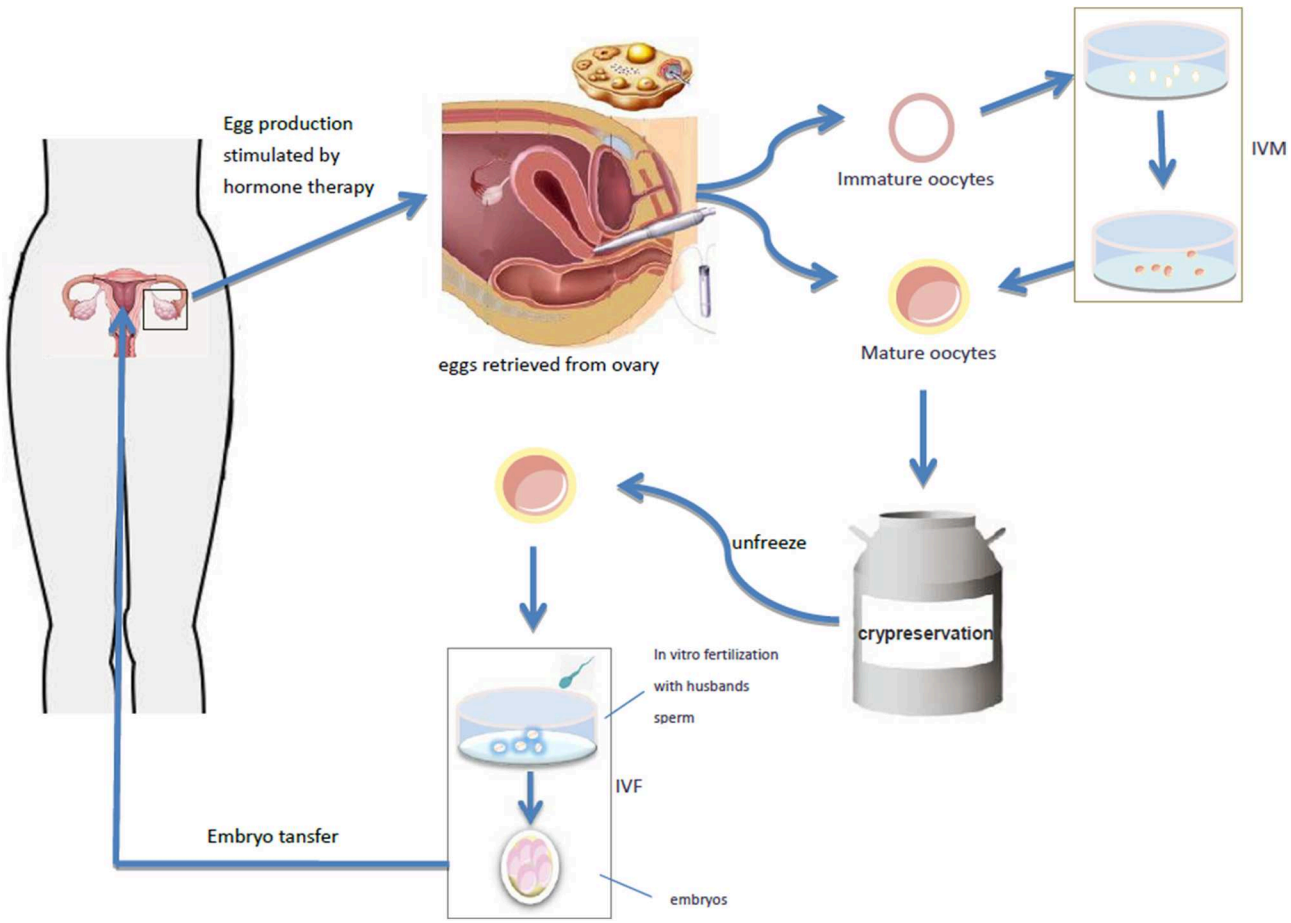

FIGURE 1 | The follicular maturation is monitored by ultrasound, and then the oocyte is collected by ultrasound guided ovum retrieval. The collected oocytes are vitrified and cryopreserved, and the frozen oocytes will be thawed and fertilized at the appropriate future time. It takes out the immature oocytes from the ovary and puts them into the culture medium simulating the follicular microenvironment *in vivo*, and cultures the oocytes *in vitro* to mature stage, and then fertilization and pregnancy occurs through IVF-ET. Oocyte maturation can be directly performed by IVF-ET and pregnancy.

TS. Ovarian tissue transplantation is the most appropriate option for prepubertal girls with TS and merits further investigation.

Cryopreservation of Ovarian Tissue

This technique requires laparoscopic surgery to obtain the ovarian cortex. Because the ovarian reserve of women with TS is low and the number of follicles in the ovary is small, to improve the success rate, it is recommended to obtain as much ovarian cortex as possible, sometimes even part or the whole ovary. Ovarian cortex containing follicles was cryopreserved. The cryopreserved ovarian tissue is thawed when women with TS are ready for pregnancy. The location of ovarian transplantation depends on whether the patient retains part of the ovary when the ovarian cortex is removed. If the ovary is retained, it can be orthotopically transplanted to the ovarian remnant or mesovarium; if no ovary exists, it can be transplanted to the site where the pelvic vessels are more abundant. Mamsen et al. (11) recently reported a total of 15 women with TS aged 5.0–22.4 years old who underwent ovarian tissue cryopreservation. A substantial number of follicles was only present in girls with mosaic TS.

As the primum movens of ovarian failure in women with TS is an abnormal apoptotic cycle. The success of cryopreservation and transplantation of ovaries depends on the number of oocytes in the transplanted tissues. As the number of primitive follicles in the ovaries of TS girls is relatively small, and some oocytes may be damaged during cryopreservation, the success rate of cryopreservation in women with TS may be relatively low. In addition, the increased apoptotic cycle will resume once the grafts begin their activity, quickly diminishing the available oocytes. To improve the results, one major need is to improve graft revascularization. Some studies showed that stimulation by angiogenic and anti-apoptotic factors, such as encapsulated vascular endothelial growth factor (VEGF) or stromal cells enriched in CD34 cells before transplantation may substantially contribute to angiogenesis (31, 36–38). In conclusion, ovarian tissue cryopreservation may be applicable to women with TS who have sufficient ovarian reserve but are not sufficiently mature to cryopreserve oocytes. So far there are no reported

cases of women with TS achieving successful pregnancy through this technique.

Ovarian Activation *in vitro*

In vitro ovarian activation (IVA) is a technique in which signal pathway activators are used to process ovarian tissues *in vitro* to activate primitive follicles in the ovary. Kawamura et al. (39) showed that ovarian fragments may interfere with the ovarian Hippo signaling pathway and lead to the growth of ovarian follicles. Based on the destruction of Hippo signal and AKT stimulation, primitive follicles were activated before ovarian tissue transplantation to promote the growth of follicles, restore mature oocytes, and achieve pregnancy by IVF-ET. The activated ovarian tissue can then be transplanted into the patient to increase the success rate of the ovarian transplantation (**Figure 2**). The first IVA baby in the world was born in Japan in 2013 (40). So far, only two cases of successful pregnancy using this technique have been reported, but none in TS, and it is still in an experimental stage (41). It can be hypothesized that it can be successfully used for women with TS with ovarian tissue transplantation and help improve their pregnancy success rate.

Studies have shown that taking multiple biopsy specimens from one ovary does not affect future hormone production, but a single ovary removal by ovariectomy can expedite menopause by 1–2 years (42). Theoretically, it may also accelerate the onset of premature ovarian failure in women with TS. Nevertheless, this technology also offers hope for fertility preservation in women with TS. Cryopreservation of ovarian tissue may be the only way to achieve fertility in some women with TS, whose ovaries cannot wait until puberty for oocyte cryopreservation, and it can even be performed in young children with TS.

Oocyte Donation and Embryo Cryopreservation

In most women with TS, ovarian function begins to decline before puberty, and by the time many of the patients desire to get pregnant, ovaries are failing, though follicles may still exist. There is little hope for pregnancy, through ovarian stimulation and *in vitro* fertilization and all above mentioned techniques are still experimental. At that point, the best option to achieve pregnancy is by oocyte donation. Deligeoroglou et al. (43) reported three out of four patients diagnosed with TS who underwent of *In Vitro* Fertilization (ICSI-IVF) with donor oocytes and brought pregnancy to completion. They found that women with TS could get the possibility to get pregnant only when diagnosed in childhood or adolescence and undergoing hormone replacement therapy (HRT). Pregnancy rates using assisted reproduction techniques (ART) and -primarily- with donated oocytes are better in women with TS who have mature secondary sexual characteristics and almost normal size uterus. In a case report of 2008, a mother donated her oocytes to her own TS daughter and had them cryopreserved for future use (44). Letur (45) reported that 18% of women with TS benefited from this technology in 2007. Embryo cryopreservation technology is one of the most common cryopreservation techniques in assisted reproductive technology. Most women with TS have reportedly used donor oocytes for *in vitro* fertilization (IVF-DO) and successful pregnancy, with a success rate of ∼37.8% (46). Andre et al. (47) reported that from 2012 to 2016, 73 women with TS obtained oocytes from 10 oocytes donation centers in France, resulting in 39 successful pregnancies after embryo transfer. However, IVF-DO has not been implemented in many countries due to ethical disputes and legal issues. For example, it is allowed by law in United States, Argentina, Russia, etc., but there is currently no relevant legal support in China.

PREGNANCY IN TURNER SYNDROME AND COMPLICATIONS OF PREGNANCY

For women with TS, there are more maternal and fetal complications irrespective of the method of conception. In 2019, Andre et al. (47) reported 151 cases of embryo transfer and 39 cases of gestation in 73 TS patients, including 24 cases of healthy delivery, 11 cases of spontaneous abortion, 3 cases of artificial abortion, 1 case of ectopic pregnancy, 1 case of non-cardiovascular death due to gestational hypertension. Moreover, the risk of spontaneous abortion is higher, mainly due to genetic abnormalities of the fetus. Intrauterine growth retardation, low birth weight, premature delivery, and stillbirths, possibly due to poor uterine environment may also occur. Women with TS who plan to pursue pregnancy need comprehensive screening and counseling before conception, including cardiac function assessment, blood pressure monitoring, echocardiography, chest MRI, abdominal ultrasound, etc. to fully understand and assess the pregnancy risk. The two most important points are prenatal genetic screening to avoid embryo chromosomal abnormalities, and pre-pregnancy cardiac assessment. Given that the cardiac output of pregnant women is about 50% higher than that of non-pregnant women and 23–50% of women with TS have congenital heart disease, the most common type being the two-leaf aortic valve (48). Because of contraction and dilatation of the aorta, pregnancy significantly increases the risk of cardiovascular complications associated with TS. Pregnancies in women with TS are at higher risk of maternal death from aortic dissection or dissection rupture or tear at the aortic root and are also at high risk of serious hypertensive disorders regardless of the use of autologous or donated oocytes (49). Hadnott et al. report, the incidence of aortic dissection in women with TS after pregnancy can be as high as 2.0%∼4.8 and the risk of developing preeclampsia is about 21% (20, 50). Therefore, pre-pregnancy cardiac assessment with measurement of the aortic size index (ASI) is indispensable for women with TS who are considering pregnancy. An ASI index of >2 cm/m^2 is considered a contraindication to pregnancy (51). In addition, because of the cephalopelvic disproportion in TS women, the cesarean section rate is higher, followed by the increased risks associated with cesarean section. In short, all women with TS who intend to carry a pregnancy should be informed of these possible risks in advance, should be evaluated before pregnancy and be closely monitored regularly during pregnancy and postpartum. Furthermore, considering the risk of pregnancy in women with TS, it is recommended to transfer only one embryo, should

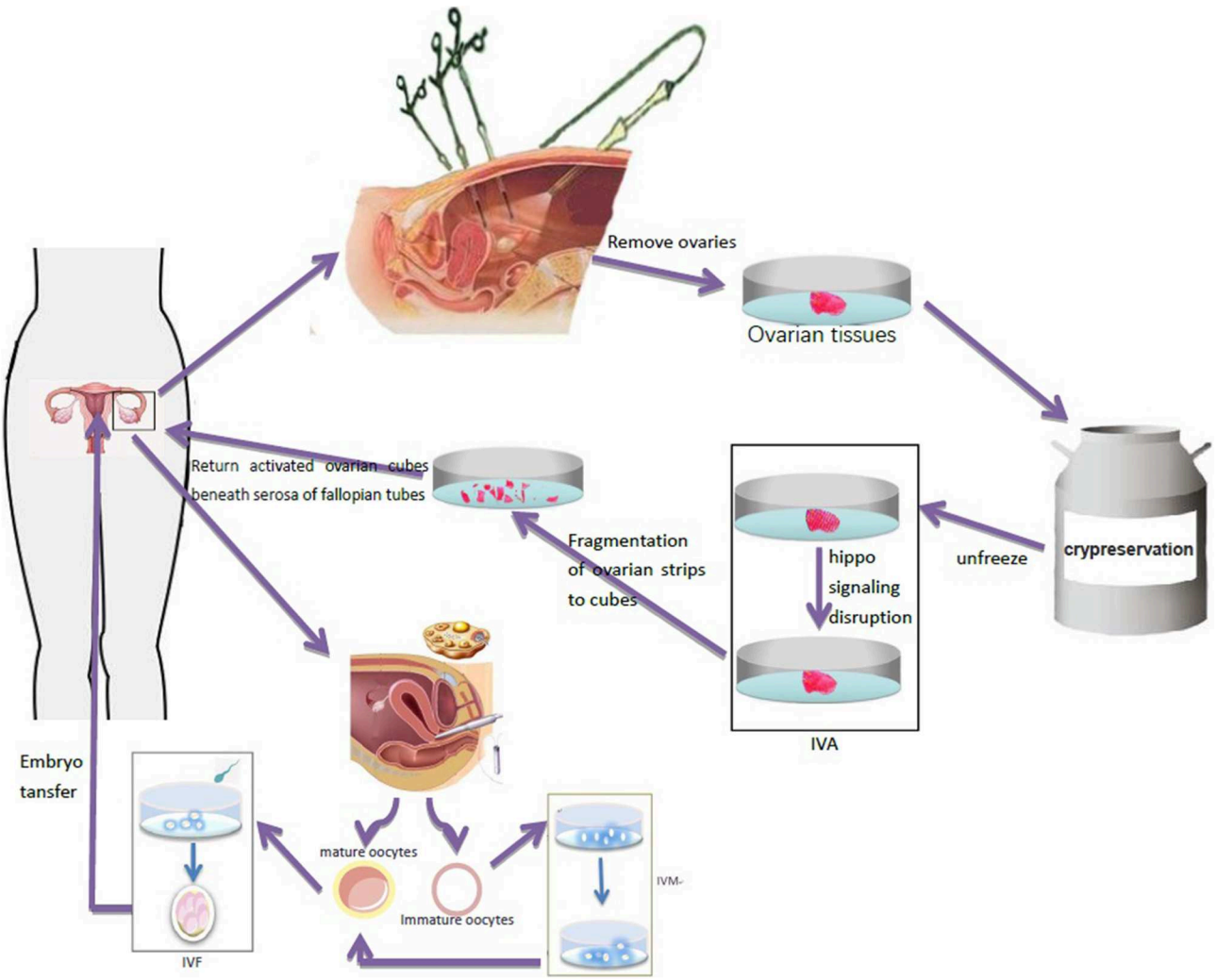

FIGURE 2 | Ovarian tissue was removed by laparoscopy and frozen for preservation. After thawing, ovary fragmentation and autologous transplantation after Akt stimulation were performed to promote the growth of follicles and generate oocytes in TS patients. Immature oocytes were cultured *in vitro* to mature oocytes, which were used for IVF-ET and pregnancy.

IVF-ET be performed, in order to minimize the risk of multiple pregnancy (49).

SURROGACY AND ADOPTION

Gestational surrogacy refers to the procedure by which accepts to become pregnant and give birth to a child not biologically related to her for an intended woman/couple. The source of oocytes can be autologous or donated. Because of the high risks of serious cardiovascular disease and other complications during pregnancy in women with TS, gestational surrogacy is a reasonable alternative to pregnancy in the few countries where surrogacy is legal. However, gestational surrogacy is not allowed due to lack of relevant laws in many countries, e.g., many Asian countries, especially China, but it is allowed in the United States, Russia, Ukraine, and so on. Adoption is feasible for all women with TS, and this method, as well, completely bypasses the risks of the major complications associated with a TS pregnancy.

CONCLUSIONS

In summary, the risks of premature ovarian failure and infertility in TS are extremely high, as the ovarian reserve in girls with TS will be already exhausted before adulthood. In order to maximize the benefits of fertility preservation, it is recommended that all women with TS should be diagnosed as early as possible, evaluated for ovarian reserve, and be offered options for fertility preservation in case of residual ovarian function. Cryopreservation of oocytes and embryos are two well-established methods of fertility preservation available for women with TS. Cryopreservation of ovarian tissue is still in experimental stage, but appears to be a promising

technique, especially if accompanied with the ovarian activation *in vitro* technique. For those women with TS who have lost their ovarian reserve, oocyte or embryo donation and adoption can be a way to fulfill their childcare aspirations. The risks of spontaneous abortion, fetal abnormalities, maternal complications, and mortality in women with TS are much higher than those in women with normal karyotypes. Patients with TS with pregnancy contraindications can use their own or donors' oocytes or embryos for gestational surrogacy.

AUTHOR CONTRIBUTIONS

MY wrote the article and searched the literature. JY and IK reviewed the literature and contributed to the writing of the article. LL contributed to the conceiving and final editing of the article.

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Pregnancy at 40 years Old and Above: Obstetrical, Fetal, and Neonatal Outcomes. Is Age an Independent Risk Factor for Those Complications?

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Objectives: Maternal age has been increasing for several decades with many of these late pregnancies between 40 and 45 years old. The main objective of this study is to assess whether maternal age is an independent factor of obstetric, fetal, and neonatal complications.

Patients and methods: A monocentric, French study "exposed-unexposed" was conducted during 11 years in a maternity level IIB. Maternal and perinatal outcomes were studied using univariates and multivariate analysis. We compared women aged 40 and above in a 1:1 ratio with women of 25–35 years old.

Results: One thousand nine hundred eighty-two women were 40 or older (mean age: 41.9) on the day of their delivery and compared to other 1,982 women who were aged between 25 and 35 years old (mean age: 30.7) Preeclampsia, gestational diabetes, were significantly higher in the study group (4.6 vs. 1.5% and 14.5 vs. 6.9%, respectively, $p < 0.001$). We found also a significant difference for gestational hypertension (3.1 vs. 1.1% $p < 0.001$), preterm birth (10.4 vs. 6.5% $p < 0.001$), cesarean (16.6 vs. 5.4% for scheduled cesarean, and 50.4 vs. 13.9% for emergency cesarean, $p < 0.001$) and fetal death *in utero* (2.1 vs. 0.5% in the study group, $p < 0.001$). These results were also significantly different in multivariate analysis.

Conclusion: A pregnancy after 40 years old is worth considering today as far as the risk factors are controlled and understand by the patient and the obstetrician. However, they have a significantly higher risks of cesarean, preterm delivery, pre-eclampsia, gestational diabetes, and fetal death *in utero* (FDIU). It is therefore the responsibility of the obstetrician to inform correctly these women in a detailed way, to reassure them and to adapt the monitoring of their pregnancy accordingly.

Keywords: advanced maternal age, complication, neonatal, pregnancy, preeclampsia, gestationnal diabetes

INTRODUCTION

Late pregnancies have been a sensitive subject in society and in the medical field since a couple years. Indeed, maternal age has been increasing for several decades with many of these late pregnancies between 40 and 45 years old (1). In France, the latest INSEE report shows that the proportion of pregnant women over 35 years rose from 19.3 to 21.3% between 2010 and 2016. This report states that about 5% of women who give birth are 40 years old or older. The age of first pregnancy increased from 29.5 in 2003 to 30.4 in 2016 (2–4). Decades earlier, a pregnancy was considered "late" if it was obtained after 35 years, today the threshold has shifted to 40 years or even 43 or 45 years according to the scientific literature (3, 5–7). This is explained by a societal evolution marked by a constantly increasing level of studies by women who have more responsibilities at work and therefore delay their project of childbearing giving their first priority to their professional career (1). In addition, the increasing development of medically assisted procreation (ART), particularly with access to oocyte donation in European countries, has recently been re-established (6, 8, 9).

According to many studies, advanced maternal age is often associated with several obstetrical complications (gestational diabetes, hypertension, pre-eclampsia (1–3, 5, 6, 10), and fetal complications (growth retardation, prematurity, fetal malformation) (1–3, 5, 6, 10).

However, the ratio of maternal mortality (RMM) remains stable at 10.3/100,000 births, according to the 5th report of the national perinatal survey on maternal deaths in France.

The age of women is an uncontested factor of maternal death risk (11). Between 2010 and 2012, nearly 30% of maternal deaths occurred in women aged 35–39 years old (for 17% of live births in this age group), vs. 10% for women aged 40 and over (for 5% of live births in this age group) (2). Today, maternal complications are managed more effectively than some years ago. Maternal mortality in France is 80 women per year. Given the small numbers, these evolutions should be interpreted with caution.

Thus, many studies suggest that maternal age increases the risk of these complications, but sometimes not statistically significantly because of small samples (3).

Moreover, there are few studies taking into account the very advanced maternal age considered in the literature around 43 and 45 years old (4, 9, 12, 13).

Studies are still few and obstetric and pre-conceptional monitoring of these patients is not yet standardized.

Therefore, we decided to carry out an additional study with a sample of women over 40 years old to confirm this trend.

The main objective of this study is to assess whether maternal age is an independent factor of obstetric, fetal, and neonatal complications.

PATIENTS AND METHODS

Design of the Study and Participants

This is a French cohort "exposed – unexposed," monocentric study performed in a maternity level IIB (hospital Foch, Suresnes). All women aged 40 or older who gave birth or who had a late miscarriage or medical termination between January 1, 2006 and December 31, 2017 were extracted from the DIAMM database and included in the exposed group. The unexposed group was constituted as follows: each woman aged 40 or over was matched with a woman aged 25–35, for whom delivery or late miscarriage or medical termination of pregnancy was performed consecutively (delivery number).

The characteristics of each pregnancy were extracted from the DIAMM software: maternal characteristics: medical history (High Blood Pressure—Type I or II diabetes—thromboembolic disease (defined as any history of venous or arterial thrombosis and/or the presence of anti-phospholipid syndrome), parity, body mass index -BMI-, smoking, geographical origin, assisted reproduction technology (ART) (artificial insemination, IVF, ICSI, gamete donation), type of pregnancy (single or twin), the method of delivery, and indications for cesarean section (CS).

Adjustment factors for preeclampsia are BMI, presence of at least one medical history, mode of conception and type of pregnancy. For gestational diabetes, the adjustment was made on BMI, the mode of conception and the type of pregnancy. For pregnancy HTA, the adjustment was made on BMI and type of pregnancy. For prematurity the adjustment factors were parity, ethnic group, type of pregnancy, pre-eclampsia, and gestational hypertension. Finally, for the variable "cesarean section" the adjustment was made for BMI, geographic origin, conception mode, and type of pregnancy.

Objectives

The main objective of the study is to determine the incidence of obstetric, fetal, and neonatal complication and to assess whether age is an independent factor of these complications.

The secondary objectives are to determine whether there is an association between some complications (pre-eclampsia, gestational diabetes, prematurity) and the conception mode associated with the type of pregnancy (singleton or twin).

The obstetrical complications studied are gestational hypertension (defined as systolic >140 mmH and/or diastolic >90 mmHg without proteinuria), pre-eclampsia (systolic >140 mmHand/or diastolic >90 mmHg associated with a proteinuria of 24 h >300 mg), gestational diabetes (defined according to the recommendations of the 2015 CNGOF), cesarean section (CS), admission of women to the intensive care unit during their pregnancies, postpartum hemorrhage (loss of more than 500 cc of blood within 24 h after vaginal delivery or CS) and blood transfusion.

The fetal complications studied are intrauterine growth retardation (IUGR) (defined as having an estimation of fetal weight <5e p) and fetal death *in utero* (FDIU).

The neonatal complications studied were prematurity (birth before 37 weeks), pH at birth (acidosis with pH <7.10), APGAR score (<7), and pediatric care just after the birth.

STATISTICS

Continuous variables are reported as mean ± standard deviation (sd). Categorical variables are reported as number and percentage (percentages were calculated excluding missing data) and are

compared by Chi-2 test or Fisher's exact test, as appropriate. Missing data were not treated.

Incidence of each complication (obstetric, fetal, or neonatal) was presented with its associated 95% confidence interval (Wilson method). The association between age and obstetric, fetal, or neonatal complications was assessed using univariate analysis. When age was significantly associated with a complication ($p < 0.05$), a multivariate analysis was performed adjusted for mother and/or pregnancy characteristics significantly associated with this complication in univariate analysis ($p < 0.2$). Univariate characteristics tested were, BMI, geographical, smoking, parity, presence of at least one medical history (hypertension, diabetes, venous thromboembolic disease/vascular pathology/lupus), mode of conception, type of pregnancy, delivery period (2006–2009 vs. 2010–2017) and for prematurity, gestational diabetes, pregnancy-induced hypertension, and preeclampsia. Multivariate analyses were performed using a logistic regression model (stepwise selection). The results are interpreted in terms of adjusted odd ratios with their associated 95% confidence interval.

A $p < 0.05$ was considered significant unless otherwise specified. All statistical analyses were performed with SAS release 9.4 (SAS Institute Inc, Cary, NC) statistical software package.

RESULTS

Thirty-two thousand two hundred forty-three patients gave birth between January 2006 and December 2017, of whom 1,982 were 40 or older on the day of delivery (**Figure 1**). The rate of late pregnancies in our study doubled from 2006 to 2016.

The demographic and obstetric characteristics for the study and the control groups are given in **Table 1**. The analysis of those characteristics did not find a significant difference between the two groups concerning: ethnic group, medical history, or smoking. There was a significant difference between the two groups for the access to ART, Body mass index (BMI), and type of pregnancy ($p < 0.001$) (**Table 1**).

Table 2 compares obstetric, fetal, and neonatal complications in univariate analysis. There is a significantly higher rate of obstetric pathology with 4.6% of pre-eclampsia for women aged 40 and over compared with 1.5% in the control group and 3.1 vs. 1.1% for gestational hypertension. There is also a significant difference for transfusion. With regard to gestational diabetes, there was 14.5% of women aged 40 and over, compared to 6.9%. However, no significant difference was found for postpartum hemorrhage and transfer to an intensive care unit. During the 11 years studied, no maternal deaths were observed.

For fetal and neonatal outcomes, there is a significantly higher proportion of FDIU, IUGR, and prematurity. There are comparable rates between the two groups for Apgar score, cord pH, and pediatric care just after birth.

Maternal and fetal complications in multivariate analysis are given in **Table 3**.

Among patients of 40 years old and above, obstetric complications are significantly more frequent, with an increased risk of gestational diabetes (OR = 2.49 (95% CI 1.61, 3.85) <0.0001), pre-eclampsia (2.46 [1.58; 3.81] <0.0001), gestational hypertension (2.59 [1.57, 4.30] 0.0002) and cesarean delivery (2.07 [1.78, 2.42] <0.0001) (**Table 3**). Fetal risk of FDIU was significantly greater in patients 40 years of age or older (OR = 4.31 95% CI [2.16, 8.60] with $p < 0.0001$). Similarly, for prematurity, where the difference observed is significant between the two groups ($p = 0.0010$).

The secondary objectives are specified in **Table 4**. There is a significant difference for preeclampsia and prematurity. No significant difference was observed for gestational diabetes. We find a rate of pre-eclampsia in patients using ART higher than in patients having a spontaneous pregnancy among the women

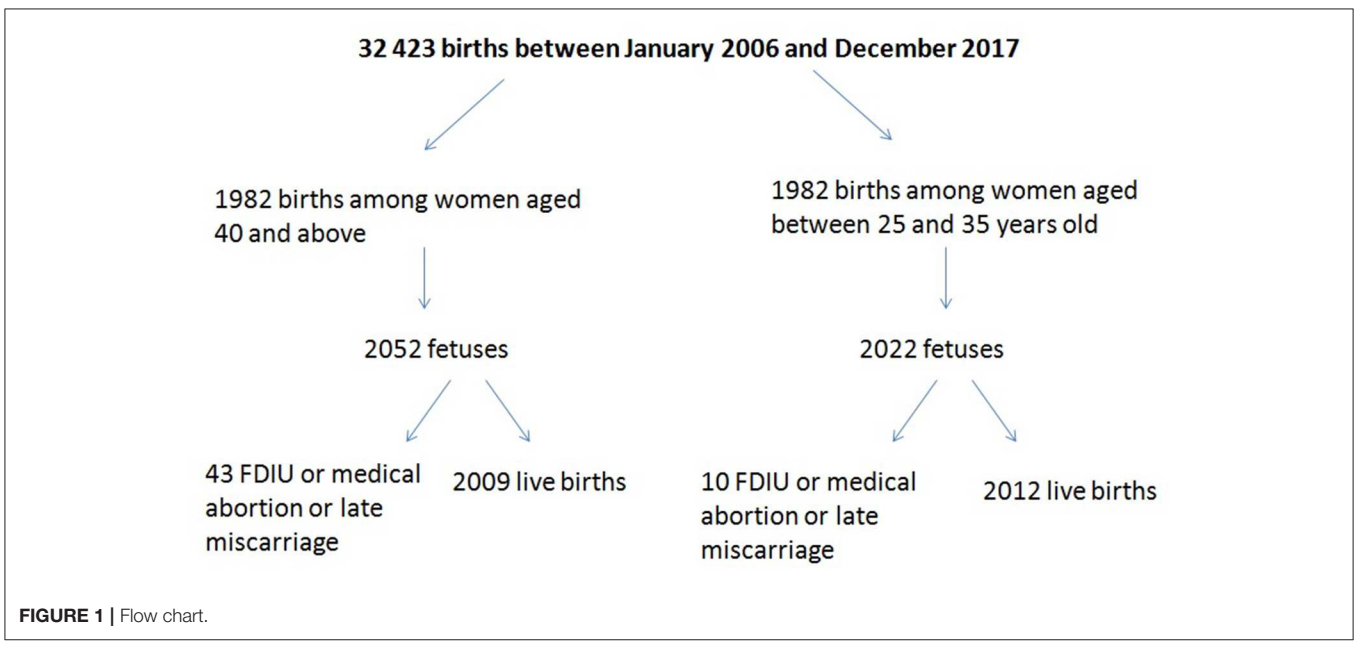

FIGURE 1 | Flow chart.

TABLE 1 | The demographic and obstetric characteristics for the study and the control groups.

	40 years and over (*N* = 1,982)	25–35 years (*N* = 1,982)	*p*
Maternal age (standard deviation)	41.9 (1.8)	30.7 (2.6)	
Ethnic group			0.115
Europe	1,272 (65.7%)	1,274 (66.5%)	
Africa	530 (27.4%)	478 (24.9%)	
Overseas departments and territories	35 (1.8%)	46 (2.4%)	
Others	98 (5.1%)	118 (6.2%)	
BMI (kg/m2)			<0.001
- <18	51 (2.6%)	59 (3.0%)	
- [18;25]	1,336 (67.8%)	1,483 (75.0%)	
- [25;30]	398 (20.2%)	319 (16.1%)	
- [30; 35]	143 (7.3%)	87 (4.4%)	
- [35; 40]	31(1.6%)	25 (1.3%)	
- ≥40	11 (0.6%)	4 (0.2%)	
Smokers	158 (8.0%)	184 (9.3%)	0.141
Parity - primiparity	730 (36.9%)	1,444 (72.9%)	<0.001
Multifetal gestation			<0.001
- single	1,601 (81.8%)	1,868 (94.3%)	
- twins	356 (18.2%)	112 (5.7%)	
Mode of conception			
- Spontaneous pregnancy	1,601 (81.8%)	1,868 (94.3%)	
- Pregnancy with ART	356 (18.2%)	112 (5.7%)	<0.001
History of high blood pressure	107 (5.4%)	92 (4.6%)	0.272
History of diabetes	62 (3.1%)	63 (3.2%)	0.932
History of thrombo-embolic event, vascular pathology, MTEV, Lupus	43 (2.2 %)	47 (2.4%)	0.673
At least one risk factors*	195 (9.9%)	179 (9.0%)	0.379

**Any patient with hypertension and/or diabetes and/or Thromboembolic event/vascular pathology/lupus.*

TABLE 2 | Obstetrical, fetal, and neonatal outcome.

	40 years and over (*N* = 1,982)	25–35 years (*N* = 1,982)	*p****
Preeclampsia	90 (4.6 %)	30 (1.5%)	<0.001
	[3.7%; 5.6%]	[1.1%; 2.2%]	
Gestational hypertension	61 (3.1%)	21 (1.1%)	<0.001
	[2.4%; 3.9%]	[0.7%; 1.6%]	
Gestational diabetes	286 (14.5%)	136 (6.9%)	<0.001
	[13.0%; 16.1%]	[5.8%; 8.1%]	
Post-partum hemorrhage	81 (4.1%)	63 (3.2%)	0.121
	[3.3%; 5.1%]	[2.5%; 4.0%]	
Blood transfusion	17 (0.9%)	6 (0,3%)	0.021
	[0.5%; 1.4%]	[0.1%; 0.7%]	
Admission of the mother in intensive care unit	11 (0.6%)	6 (0.3%)	0.224
	[0.3%; 1.0%]	[0.1%; 0.7%]	
Delivery mode			<0.001
- Scheduled cesarean	328 (16.6%)	108 (5.4%)	
	[15.0%; 18.3%]	[4.5%; 6.5%]	
- Emergency cesarean	404 (20.4%)	275 (13.9%)	
	[18.7%; 22.2%]	[12.4%; 15.5%]	
- Vaginal delivery	1249 (63.0%)	1599 (80.7%)	
Prematurity (<37 wk)	206 (10.4%)	128 (6.5%)	<0.001
	[9.1%; 11.8%]	[5.5%; 7.6%]	
IUGR or fetal malformation ¤	167 (8.3%)	133 (6.6%)	0.039
	[7.2%; 9.6%]	[5.6%; 7.8%]	
Apgar score less or equal to < 7¤	43 (2.1%)	38 (1.9%)	0.573
	[1.6%; 2.9%]	[1.4%; 2.6%]	
Neonatal pH in the umbilical cord<7,10¤	82 (4.3%)	90 (4.6%)	0.591
	[3.5%; 5.3%]	[3.8%; 5.7%]	
New-born intensive care¤	234 (11.7%)	197 (9.8%)	0.054
	[10.3%; 13.2%]	[8.6%; 11.2%]	
Fetal death *in utero**	43 (2.1%)	10 (0.5%)	<0.001
	[1.6%; 2.8%]	[0.3%; 0.9%]	

** in 2052 fetuses for over 40s and 2022 fetuses in 25–35 years ¤on 2009 fetuses for over 40s and on 2012 fetuses on 25–35s *** Chi-2 test.*

of 40 and above (for multifetal gestation and ART 22.5 vs. 16.7% and single pregnancy with ART 6.6 vs. 3.3%). We did not find a significant increase in gestational diabetes regardless of the type of conception (ART vs. spontaneous pregnancy).

DISCUSSION

Our study shows that advanced maternal age is an independent risk factor for obstetric and neonatal complications (14, 15). In fact, multivariate analysis found significant results for three of the most common pregnancy-related diseases: gestational hypertension, pre-eclampsia, and gestational diabetes. Our large sample significantly confirms the occurrence of pre-eclampsia in women aged 40 and above, unlike some studies with small samples that did not find this result in multivariate analysis (3).

Moreover, there is a higher risk of pre-eclampsia when the patient has some other risk factor such as twin pregnancy or medical history (hypertension and/or diabetes and/or VTE/vascular disease/lupus) (16, 17). Even more, these women with advanced maternal age are at higher risk of developing cardiovascular and nephrological diseases in the long term (18). In the case of tobacco, it has not been found as an independent risk factor, which can probably be explained by a significant underestimation of women reporting smoking during pregnancy.

The high proportion of cesareans in the study group of women over 40 is due to some contributing factors. On one hand, the percentage of scheduled cesareans is higher because there is a higher prevalence of uni or multi-cicatricial uterus.

Cesareans for high maternal age or for maternal request finally represented a small sample (22 women/1,982, 1% in the exposed group vs. 1/1,982 in the unexposed group). There was also a higher rate about the emergency cesareans deliveries in the study group over 40 years old. Several physiological hypotheses have been mentioned in previous studies (2, 3): a higher rate of dystocia presentation and scarred uterus, uterine contractility

less effective than for a woman aged 25–35. In our sample, the most common indications for CS were abnormalities of cardio-fetal rhythm and cervical dystocia (19). It is likely that CFR abnormalities are more severely judged by the obstetrician, in the context of older patients, especially if the pregnancy is a result of ART, putting some women at a risk of cesarean that is not always justified (20). In total, this large proportion of CS in women 40 years and older has also been shown in other studies (3, 4, 17, 20–22). However, these results should be taken with caution because some indications for CS are inherent to the protocols practiced in our unit.

The association between advanced maternal age and fetal deaths *in utero* should also be taken into account. Among those 43 FDIUs, we have looked at every medical files of those women and we did not find any events that could explained this high number. Indeed, among the 43 FDIUs in women aged 40 and above, there were no more patients using ART, nor more patients with obstetric pathology. This can be explained by a small number of FDIU. The only common point in our study group was the advanced maternal age. In these circumstances, instead of worrying the patients, it might be more appropriate to give them clear and reassuring information while performing a pre-conception close monitoring and throughout the pregnancy. This would help detect and manage these complications much earlier. In addition, with the advanced technology, several risks are now monitored using non-invasive prenatal screening or even the pre-implantation diagnosis (23–26).

The incidence of maternal complications is likely to increase over time due to increased maternal age. It will be difficult to reduce the incidence of these complications, but we can reduce the serious complications of preeclampsia, gestational diabetes (such as eclampsia, and macrosomia) through appropriate management (induce delivery before 41 weeks, close monitoring of the fetus) (27, 28).

With regard to neonatal complications, few significant differences were found in our study, as well as in the literature (29, 30). This is partly explained by the fact that several obstetrical factors can interfere without being related to age (the length of the delivery, abnormalities of the RCF, chorioamnionitis (3, 31, 32).

Our study has several advantages. On the one hand, our study was done on a large sample, with data processing from medical records with a complete search for missing data. International and European studies with large samples use public health registers, thereby providing a lot of information on the characteristics of the population (5, 10). However, this is often at the expense of information such as the type of delivery, the methods of neonatology care which are sometimes different in hospitals.

On the other hand, we took a period of 11 years, to check if there had been a difference in daily practices. We did not notice any difference between the periods 2006–2010 and 2011–2017 except for the increasing number of patients who have access to ART.

On the other hand, we matched each patient aged 40 and above to a patient aged between 25 and 35 whose delivery number followed the patient case. Indeed, this allowed us to limit as far as possible all the variability of practices

TABLE 3 | Association between age and complications (uni and multivariate analysis).

	Univariate **OR [IC 95%] (*p*-value)**	**Multivariate**** **OR ajusté [IC 95%] (*p*-value)**
Preeclampsia	3.10 [2.04; 4.71] (<0.0001)	2.46 [1.58; 3.81] (<0.0001)
Gestational diabetes	2.30 [1.85; 2.85] (<0.0001)	2.49 [1.61; 3.85] (<0.0001)
Gestational hypertension	2.98 [1.81; 4.91] (<0.0001)	2.59 [1.57; 4.30] (0.0002)
Preterm birth <37SA	1.68 [1.34; 2.11] (<0.0001)	1.55 [1.19; 2.02] (0.0010)
Cesarean	2.35 [2.03; 2.71] (<0.0001)	2.07 [1.78; 2.42] (<0.0001)
Fetal death *in utero*	4.31 [2.16; 8.60] (<0,0001)	4,59 [2.20; 9.55] (<0,0001)

*Selection of variables p ≤ 0.15 **Stepwise model (input threshold 0.15, output threshold 0.05) Note: the multivariate model was performed on 3,821 pregnancies (143 missing data).*

TABLE 4 | Description of obstetric complications in patients aged 40 and above, type of pregnancy and use of PMA.

	Multifetal gestation and ART (*N* = 40)	**Single pregnancy and ART (*N* = 316)**	**Multifetal gestation without ART (*N* = 30)**	**Single pregnancy without ART (*N* = 1,571)**	***p***
Preeclampsia	9 (22.5%)	21 (6.6%)	5 (16.7%)	52 (3.3%)	<0.001 (F)
OR [95% IC]*	8.48 [3.84; 18.72]	2.08 [1.23; 3.50]	5.84 [2.15; 15.87]	1	
Gestational diabetes	7 (17.5%)	52 (16.5%)	7 (23.3%)	217 (13.8%)	0.242 (F)
OR [95%IC]**	1.32 [0.58; 3.03]	1.23 [0.88; 1.71]	1.90 [0.80; 4.47]	1	
Preterm<37wk	22 (55.0%)	38 (12.0%)	14 (46.7%)	126 (8.0%)	<0.001 (F)
OR [95%IC]***	14.02 [7.33; 26.82]	1.57 [1.07; 2.30]	10.04 [4.79; 21.03]	1	

**Association Pre-eclampsia and Type of Pregnancy and ART in women of 40 years and older.*

***Association Gestational Diabetes and Pregnancy Type and ART in Women of 40 Years and above.*

****Association Gestational Age <37 Weeks and Type of Pregnancy and ART in Women Age 40 and above.*

on the delivery route (natural delivery vs. cesarean, neonatal care). We also had the advantage of separating fetuses, newborns, and mothers, which has not been realized in other studies, and which may lead to a classification bias regarding perinatal outcomes.

Our study is yet limited by its monocentric character and retrospective aspect. In addition, Foch Hospital has an ART center, so our sample probably contained more patients using these techniques. However, we had the opportunity to have 18.2% of women over 40 using ART. This allowed us to highlight the significant increase in preeclampsia and prematurity in patients over 40 years of age who have used ART. After 44 years, 1 out of 2 women used the ART. This rate is surely underestimated because there is a large number of patients who voluntarily omit to declare their use of ART in particular the use of donated oocytes (33).

It is especially remembered that maternal complications occurring decades ago are less morbid today than before (22, 34). Screening and management of maternal and neonatal complications are progressively improving, and a high-risk pregnancy at age 40 in the 1980s should no longer discourage patients and obstetricians in 2020.

CONCLUSION

The desire to become pregnant after 40, 45, or even 50 years will most likely continue to develop in our society. Thus, it is important to multiply the studies on this subject in order to inform these patients as much as possible. Our study confirms the current trend among this group of women over 40 years of age to know that advanced maternal age leads to more significant obstetric complications.

However, in the absence of cumulative risk factors and with appropriate management, a pregnancy after 40 years can go well-physiologically without having a higher maternal mortality or neonatal morbidity.

It is therefore the duty of the obstetrician to inform these women in an enlightened way, to reassure them and to adapt the monitoring of their pregnancy according to the risk factors, the modality of conception, and the multifetal gestation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

AB and IA created and organized the database and wrote several sections of the manuscript. JA, HA, MC, and PP contributed conception and design of the study. SR performed the statistical analysis and wrote the statistics section of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Impact of Ovarian Yield—Number of Total and Mature Oocytes Per Antral Follicular Count—On Live Birth Occurrence After IVF Treatment

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To assess the relation between oocytes yield including total retrieved oocytes (O)c and total mature oocytes (MII) relative to the antral follicular count (AFC) (3–9 mm in diameter) and relative to anti-müllerian hormone (AMH) ng/mL level: Oc/AFC, MII/AFC, Oc/AMH, and MII/AMH, respectively, and ART outcomes. We included retrospectively 264 IVF cycles after the first embryo transfer (ET) and after the cumulative ET (CET). The implantation rate (IR) and the live birth rate (LBR) after first ET were 31 ± 39% and 32.6%, respectively, and after CET 35 ± 38% and 45.1%, respectively. There was a significantly higher average of Oc/AFC and MII/AFC when live birth (LB) occurred after the first ET (0.82 ± 0.4 vs. 0.71 ± 0.35 and 0.57 ± 0.4 vs. 0.68 ± 0.3, respectively, $P < 0.05$). We reported a significantly higher average of MII/AFC when LB occurred after CET (0.66 ± 0.3 vs. 0.56 ± 0.30, $P = 0.02$) in comparison to the group where no LB was obtained. Increased Oc/AFC and MII/AFC ratios were associated with the occurrence of LB and increased IR after first ET ($P < 0.05$). Increased MII/AFC ratio was associated with the occurrence of LB and IR after CET ($P = 0.02$ and $P = 0.04$, respectively). After age-adjusted multivariate analyses, all these trends were confirmed ($P < 0.05$) except for the effect of MII/AFC ratio on IR after CET. In conclusion, Oc/AMH and MII/AMH ratios have no effect on the occurrence of LBR or on IR after first ET or CET at either age grouping. Ratios Oc/AFC and MII/AFC seem promising indicators to assess ovarian response.

Keywords: ovarian yield, AFC, AMH, follicular output rate, IVF outcome, oocyte index

INTRODUCTION

ART treatments are based on ovarian stimulation (OS), for which the use of gonadotropins remains essential. Despite years of experience, the choice of the optimal dose of gonadotropins varies considerably depending on physicians' experience and the protocols established by each center. The main objective of OS is to induce multiple ovulation in order to retrieve several mature oocyte and obtain several embryos available for transfer, thus increasing ART efficiency (1–3).

In current practice, physicians adjust OS parameters by assessing ovarian reserve, including an antral follicular count (AFC), a measure of serum anti-müllerian hormone (AMH) level and baseline (day-3) serum follicle-stimulating hormone level before starting OS (4). Although all

of these tests provide information to anticipate response to OS, the AMH level is the most widely used in routine practice (5–7).

Retrospective analysis of a cycle of ovarian stimulation makes it possible to evaluate the ovarian response of each patient, even if this response may vary from one cycle to another. This is why some authors describe several categories of patients according to their response to ovarian stimulation: poor responders if they obtained <3 oocytes at oocyte retrieval, suboptimal responders between 4 and 9 oocytes, normo-responders between 10 and 15 oocytes and hyper-responders for those who obtain more than 15 oocytes (8, 9).

Currently, two main parameters are recognized to predict response to ovarian stimulation by many authors: (i) plasma AMH level (10, 11) and (ii) AFC (12, 13). However, in practice, the predictive value of these two parameters may have limitations and the ovarian response may not correspond to the expected response. Therefore, the ability of these two ovarian markers to reflect oocyte quality and competence is still debated and controversial (4, 12, 13).

As a result, it is important and timely to provide simple, reproducible ovarian response analysis tools that can predict the chances of success of ART treatment.

Some authors suggested that the ovarian yield—antral follicle responsiveness to follicle-stimulating hormone administration—could be a good standardized tool for evaluating the response to stimulation and predicting ART outcome because it would take into account the baseline ovarian reserve and the final result of OS and therefore the actual ovarian potential of each patient (14). In the original study, the ovarian yield identified as the follicular output rate (FORT) index was correlated with pregnancy rates (15, 16). The FORT index was calculated as the number of pre-ovulatory follicles (16–22 mm the day of hCG injection) over the number of antral follicle (3–9 mm) at the beginning of OS. Some authors have correlated the FORT index with oocyte competence, reflected by pregnancy rates (17–19). However, while the pre-ovulatory follicles reflect the response to OS, the obtained oocytes are the concrete result of the complete procedure (stimulation and oocyte retrieval). In fact, only mature oocytes (MII) are used in IVF laboratories to obtain embryos with an implantation potential.

Further, Alviggi et al. proposed to combine the FORT index to follicle-to-oocyte (FOI) index defined by the ratio between the total number of oocytes collected and the number of antral follicles (oocyte number/antral follicle Count X100) (20). According to this publication, a normal FOI should be >50%. However, no data has shown if FOI index was correlated with pregnancy outcomes. Considering this, we investigated whether a different FOI index might correlate with IVF success rates and thus might be a more appropriate indicator of ART outcome.

The aim of our study was to evaluate the predictive value of new ovarian stimulation indicators on implantation (IR) and live birth (LB) rates. For this purpose, we decided to calculate the FOI ratio defined by the number of retrieved oocytes divided by the AFC score (Oc/AFC). To avoid the potential bias due to inter-operator variability during OS monitoring and oocyte retrieval (21), we defined the same ratios but based on the total number of mature oocytes (MII) this time (MII/AFC). Secondarily, we investigated whether the same previous ratios but using serum AMH level as denominator (Oc/AMH and MII/AMH) would be predictive of ART success.

MATERIALS AND METHODS

Study Population

We conducted a retrospective study using an anonymized IVF database, containing clinical and laboratory parameters of the Foch Hospital ART Center in Suresnes, France. All first IVF cycles carried out in our center from September 2016 to December 2017 of women aged between 18 and 43 years were potentially included. The final inclusion was possible in the absence of the following exclusion criteria: absence of one of the two ovaries, previous attempt in another center, fertilization failure, cycles with *in vitro* maturation and cycles with non-transferred frozen embryos without having obtained a LB after previous embryo transfer (ET) from the same IVF cycle.

Ethical Approval

All patients have signed an informed consent, allowing the use of their medical records for research purposes, as long as the patient's identity is protected, and data analysis is anonymized. This study was performed under retrospective protocol IRB number: 00012437 approved by the Foch hospital ethical committee.

Patient Characteristics Measurements

AMH and AFC Measurements

We collected only AMH and AFC values measured within 12 months prior to the ovarian stimulation cycle. They were obtained at time of initial patient screening during the first 3 days of the menstrual cycle. AMH value were determined by an automated multi-analysis system using a chemiluminescence technique (Architect, Abbott, Les Clayes-sous-Bois, France) or by an immune-enzymology technique (EIA Immunotech, Beckman-Coulter, France).

To determine AFC, the ovarian ultrasound scans were performed also on the first 3 days of the menstrual cycle using a 5.0–9.0 MHz multifrequency transvaginal probe (Voluson™ S10 system, GE Healthcare). We determined, at baseline, the number of all follicles measuring 2–9 mm in diameter in both ovaries.

Patient's Treatment

Ovarian Stimulation Protocol

Starting on the 20th day of previous menstrual cycle, the patients received estrogen pill (Provames® 2 mg, Sanofi Aventis, France) in an attempt to obtain a uniform follicular cohort. ART treatment followed our routine protocols. Ovarian stimulation was achieved using highly purified urinary gonadotropins: recombinant FSH, urinary FSH and/or menotropins. Individually set doses of hormones were used, ranging from 150 to 600 IU of FSH per day using an antagonist protocol. Development of ovarian follicles was monitored by transvaginal ultrasonography (TVUS) starting on the 6th day of OS. If necessary, hormonal doses were adjusted to generate an optimal response. Daily antagonist was systematically introduced

from the 6th day of OS onwards, in order to inhibit ovulation of growing follicles. During the last days of OS, patients had daily visits at our institution for TVUS and hormonal examinations in order to identify the proper timing for triggering. Final oocyte maturation was typically induced when ≥3 pre-ovulatory follicles (16–22 mm in diameter) were observed and E2 levels per pre-ovulatory follicle were >200 pg/ml. This was done using a GnRH-agonist, triptorelin at the dose of 0.2–0.3 mg (Decapeptyl®, Ibsen Pharmaceuticals, France) if there was a risk of ovarian hyper stimulation syndrome OHSS, or at dose of 0.2 mg combined with 0.25 mg of choriogonadotropin alfa (Ovitrelle®. Merck Pharmaceuticals, France) if the risk was low.

Oocyte retrieval was performed 37 h after triggering. Fertilization was achieved with either intracytoplasmic sperm injection (ICSI) or classic *in vitro* fertilization (IVFc) depending on sperm parameters. When using IVFc, oocyte maturation was assessed after denudation at day 1 after fertilization. Oocytes with one polar body before ICSI and one or two polar bodies on day 1 after IVFc were considered mature (MII).

Fresh embryo transfers (ET) were planned on day 3 or day 5 according to the medical indication. However, embryo freezing (for supernumerary embryos or as part of the differed freeze-all strategy) was always performed at blastocyst stage on day 5 or day 6. Fresh and frozen blastocyst transfers were performed if blastocyst reached a full blastocyst expansion degree with inner cell mass and trophectoderm cells compatible with a transfer (22). No PGT-A was performed.

Frozen Embryo Transfer

Endometrial preparation was achieved with a priming phase using oral E2, as follows: 4 mg/day from day 1 to 4; 6 mg/day from day 4 to 9; 8 mg/day from day 9 onwards. Endometrial thickness was monitored by TVUS, while serum E2 and progesterone were assessed in order to rule out premature ovulation prior to initiation of progesterone supplementation. Thereafter, progesterone was added using vaginal capsules (Utrogestan®, Besin Pharma, France) at the dose of 200 mg and subcutaneous injections of progesterone (Progiron®, IBSA, France) at the dose of 25 mg/day. Warmed blastocysts were transferred on the 6th day of progesterone treatment. Hormonal treatment was pursued until the pregnancy test and continued for 8 weeks if pregnant.

Ovarian Yield Calculation and Primary Endpoint

For the assessment of ovarian yield four ratios were calculated in relation to the AMH and the AFC:

- First, the ovarian yield (total retrieved oocytes and in terms of mature oocytes) relative to the AFC: Oc/AFC and MII/AFC, respectively
- Second, the ovarian yield (total retrieved oocytes and in terms of mature oocytes), this time expressed in relation to each patient's AMH ng/mL level: Oc/AMH and MII/AMH, respectively.

The primary endpoint was the occurrence of live birth (LB) after the first ET and live birth rate (LBR) was defined as the number of deliveries/total number of cycles. The secondary endpoints were the occurrence of a live birth after cumulated embryo transfers (CET), the implantation rate (IR) (defined as the total number of gestational sacs/total number of transferred embryos) after the first ET and the cumulated implantation after CET.

Statistical Analysis

Data were presented a mean with SD, or median with range, and percentage as appropriate. The Shapiro-Wilk test was performed as normality test. The association between age groups and ratios was performed using ANOVA tests.

Spearman correlations were used to evaluate the association between the implantation rate after first ET and the cumulative implantation rate with the following ratios: Oc/AMH, Oc/AFC, MII/AMH, and MII/AFC and to evaluate the correlation between AMH and AFC.

Finally, linear regression models were used to quantify the relationship between implantation rates and the different ratios. A multivariate model incorporating age as a confounding factor followed the univariate linear model.

To measure the association of the different ratios with the presence of a LB (after the first ET and cumulatively), Student tests were performed, followed by a univariate and then a multivariate logistic regression model to account for age for each ratio tested.

The tests were bilateral, the significance level set at 5% and the analyses were performed using STATA software (Statacorp L, Texas, USA).

RESULTS

Characteristics of Patients

A total of 264 IVF cycles were included in the study. Mean age in the study cohort was 35.2 ± 4.0 years. A total of 144 patients (54.5%) were under 36 old years (yo), 67 (25.4%) between 36 and 39 yo and 56 (20.1%) over 39 yo. Medical indications for ART were male factor, tubal factor, endometriosis, polycystic ovary syndrome (PCOS) and idiopathic infertility. In 62% of cases, couples presented simultaneously more than one medical indications. Mean AMH was 2.68 ± 2.65 ng/ml while mean AFC was 18.0 ± 11.2.

Out of 264 IVF cycles, 185 resulted in a fresh ET (70.1%) and 57 (21.6%) were intended to freeze-all strategy. For 22 cycles (8.3%) no embryo for transfer or freezing was obtained.

After the first ET (fresh or frozen), 103 women obtained a clinical pregnancy, among those, 17 experienced spontaneous pregnancy loss, and 86 delivered a healthy baby. The IR was 31 ± 39% and the LBR was 32.6%. After CET, we obtained 157 clinical pregnancies and 119 live births, cumulated IR and LBR were 35 ± 38 and 45.1%, respectively.

The average Oc/AFC and MII/AFC ratios were 6.6 ± 4.7 and 5.3 ± 4.0, respectively. The average Oc/AMH and MII/AMH ratios were 0.7 ± 0.4 and 0.6 ± 0.3, respectively. There was no significant difference in these four ratios according to the age categories of women. Although for the Oc/AMH ratio, we observed a tendency but not significant ($P = 0.05$) to an increase in the ratio in patients over 39 years of age (**Table 1**).

TABLE 1 | Oocyte yield indices in infertile women with or without live birth after the first embryo transfer.

	Live birth		
	Yes	No	
Ratio	Mean ± SD	Mean ± SD	*P*-value
Oc/AFC	0.82 ± 0.4	0.71 ± 0.4	**0.03**
MII/AFC	0.68 ± 0.3	0.57 ± 0.4	**0.02**
Oc/AMH	6.5 ± 5.1	6.8 ± 3.9	0.52
MII/AMH	5.57 ± 3.3	5.19 ± 4.3	0.42

Data are presented as mean with standard deviation.
Oc/AFC, total retrieved oocytes relative to antral follicular count.
MII/AFC, total mature oocytes relative to antral follicular count.
Oc/AMH, total retrieved oocytes relative to serum AMH ng/mL level.
MII/AMH, total mature oocytes relative to serum AMH ng/mL level.
Bold values for statistically significant differences.

In our population, we observed a strong positive correlation between AMH and AFC: when one increased, so did the other ($P < 0.001$).

Patient's Results

In an effort to determine whether Oc/AFC, MII/AFC, Oc/AMH, and MII/AMH ratio had an impact on the occurrence of LB and on IR in association with female age, we examined these 4 ratios using a bivariate age-grouping paradigm. The dataset was stratified according to female age, studying the oocyte yield in the following age groups: <36, [36–39], >39 years.

The Occurrence of Live Birth

When comparing the average of cycle's ratios according to the presence or absence of a LB after the first ET, there was a significantly higher average of Oc/AFC and MII/AFC for LB group (0.82 ± 0.4 vs. 0.71 ± 0.35 and 0.57 ± 0.4 vs. 0.68 ± 0.3, respectively, $P < 0.05$) (**Table 1**).

Increased Oc/AFC and MII/AFC ratios were associated with higher number of LB. Furthermore, in the age-adjusted multivariate analysis, only the MII/AFC ratio remained significant ($P = 0.04$), in contrast to the Oc/AFC ratio which had a limit *P* of 0.05 (**Table 2**).

However, the Oc/AMH and MII/AMH ratios had no effect on the occurrence of LB at either age grouping as shown in **Tables 1, 2**.

When comparing the average of cycle's ratios according to the presence or absence of a LB after CET, there was a significantly higher average of MII/AFC for LB group (0.66 ± 0.3 vs. 0.56 ± 0.30, $P = 0.02$) (**Table 3**).

Spearman's correlation analysis showed no association between Oc/AFC, Oc/AMH, and MII/AMH ratios and the occurrence of LB after cumulative embryo transfer. However, an increase in the MII/AFC ratio raised LB occurrence chances ($P = 0.02$). Which was confirmed after age-adjusted multivariate analysis ($P = 0.04$) (**Table 4**).

The Implantation Rate

There was an association between the Oc/AFC ratio and IR, and between MII/AFC ratio and the IR after the first ET: the increase in those ratios favored the increase in the IR after the first ET (OR = 1.16 IC95% [1.02–1.32], $P = 0.02$ and OR = 1.23 IC95% [1.06–1.42], $P < 0.01$, respectively). This association was found in the age-adjusted multivariate model (OR = 1.14 IC95% [1.01–1.30], $P = 0.04$ and OR = 1.20 IC95% [1.04– 1.39], $P = 0.01$). However, according to our results the Oc/AMH and MII/AMH ratios had no effect on IR at either age grouping.

Spearman's correlation analysis showed no association between Oc/AFC, Oc/AMH, and MII/AMH ratios and IR after cumulative embryo transfer. However, an increase in the MII/AFC ratio raised IR ($\rho = 0.15$, $P = 0.02$), but after age-adjusted multivariate analysis, this impact became not significant.

DISCUSSION

Our study aimed to evaluate whether the calculation of an ovarian yield according to the number of retrieved oocytes or mature oocytes, previously described to assess ovarian response, was predictive of ART success. As oocyte potential may vary according to these ratios, we chose to indirectly estimate this oocyte potential by evaluating implantation rate and live birth rate after embryo transfer. Moreover, since the embryo with the best potential within a cohort is transferred first, we chose to evaluate results after the first ET as the primary endpoint. We also chose to assess cumulative IR and LBR which provide additional information but potentially favor patients with high AFC, such as PCOS who will have statistically more supernumerary frozen embryos (8).

The advantage of those index compared to the FORT index described above is that they evaluate both the response to OS and the technical process of oocyte retrieval and recovery. It is interesting to note that FORT and MII/Oc are predictive of success in ART treatment while they are not quite comparable but possibly complementary. In fact, in the calculation of the FORT, the pre-ovulatory follicles of intermediate size (14–16 mm) are not taken into account whereas they can give mature oocytes, taken into account by our index (23).

Furthermore, while the FORT is evaluated during stimulation, and could have an impact on the decision to cancel a cycle or not, our index can only be estimated once the oocyte retrieval has been carried out, but it carries the advantage of being estimated in a more objective way (number of oocytes and number of mature oocytes) in relation to the FORT. The proposed index in this study avoids the subjectivity of ultrasound in the evaluation of the number of pre-ovulatory follicles when these are numerous, or when imaging is difficult and depends on the operator. Therefore, our proposed index could be an additional and complementary criterion to the FORT allowing to better adjust the treatment during a possible second cycle in case of failure.

As expected, we found a very strong correlation between AMH and AFC in patients. Moreover, the age of the patients had the greatest impact on implantation and birth rates (24).

The Oc/AFC ratio had a significant association with the IR, and at the limit of significance with the presence of a LB after

TABLE 2 | Odds ratios of oocyte yield indices after first embryo transfer according to age groups Naissance.

	Logistic regression			Logistic regression age-adjusted		
	OR	**IC95%**	***p***	**OR**	**IC95%**	***p***
Oc/AFC	**2.14**	**[1.08–4.21]**	**0.03**	**2.00**	**[0.99–4.01]**	**0.05**
Age (years)						<0.01
<36				1		
[36–39]				0.55	[0.3–0.99]	
>39				0.23	[0.08–0.62]	
MII/AFC	**2.50**	**[1.17–5.37]**	**0.02**	**2.28**	**[1.05–5.00]**	**0.04**
Age (years)						<0.01
<36				1	–	
[36–39]				0.55	[0.30–0.99]	
>39				0.23	[0.08–0.63]	
Oc/AMH	**1.01**	**[0.96–1.07]**	**0.56**	**1.03**	**[0.98–1.09]**	**0.24**
Age (years)						<0.01
<36				1	–	
[36–39]				0.51	[0.28–0.92]	
>39				0.20	[0.07–0.57]	
MII/AMH	**1.02**	**[0.97–1.09]**	**0.47**	**1.04**	**[0.97–1.11]**	**0.22**
Age (years)						<0.01
<36				1	–	
[36–39]				0.51	[0.28–0.93]	
>39				0.21	[0.07–0.57]	

Oc/AFC, total retrieved oocytes relative to antral follicular count.
MII/AFC, total mature oocytes relative to antral follicular count.
Oc/AMH, total retrieved oocytes relative to serum AMH ng/mL level.
MII/AMH, total mature oocytes relative to serum AMH ng/mL level.

the first ET, whatever the age of the patient. On the other hand, on the cumulative results, no association was found for this ratio. The MII/AFC ratio proved to be more influential, with a strong association with ART outcomes after first ET and a strong association on LB after cumulative transfers. These two ratios seem promising for assessing the quality of an ovarian response to gonadotropins. The fact that the ovarian yield in terms of mature oocytes is more significantly predictive than that of total oocytes is consistent with the relevance of the first one because it does not consider inter-operator variability during the oocyte retrieval procedure, and represents the expected ultimate outcome for OS, namely; obtaining mature oocytes.

In addition, MII/AFC can be a good indicator of quality in an ART center. Pirtea et al. found that the number of oocytes retrieved compared to the expected retrieved could not be used as a good key performance indicator because of its high variability (25). On the other hand, it seems to us that the number of MII can be used to estimate the effectiveness of an ART center in terms of OS protocols and oocyte retrieval efficacy.

Furthermore, in our study, no ratio using AMH in its denominator was predictive. The fact that the numerators and denominators of those ratios do not have the same units (number and ng/mL) may make them more complex and their correlation uncertain. Thus, a patient with an AMH of 0.2 ng/mL who obtains 2 MII at retrieval will have a ratio of 10, and a patient with an AMH of 1 ng/mL who obtains 10 MII will also have a ratio of 10, although we can assume that their prognosis is not the same.

TABLE 3 | Oocyte yield indices in infertile women with or without live birth after the cumulative embryo transfer.

	Live birth		
	Yes	**No**	
Ratio	**Mean ± SD**	**Mean ± SD**	***P*-value**
Oc/AFC	0.79 ± 0.4	0.72 ± 0.4	0.14
MII/AFC	0.66 ± 0.3	0.56 ± 0.3	**0.02**
Oc/AMH	6.54 ± 3.8	6.65 ± 5.4	0.84
MII/AMH	5.43 ± 3.2	5.21 ± 4.6	0.65

Data are presented as mean with standard deviation.
Oc/AFC, total retrieved oocytes relative to antral follicular count.
MII/AFC, total mature oocytes relative to antral follicular count.
Oc/AMH, total retrieved oocytes relative to serum AMH ng/mL level.
MII/AMH, total mature oocytes relative to serum AMH ng/mL level.
Bold values for statistically significant differences.

In addition, the lack of significance may be due to the lack of standardization in AMH dosing with different techniques, making its relevance uncertain, or to the complex role of AMH, which also inhibit the development of pre-antral follicles in response to FSH (26), making it inversely correlated to the FORT (16).

TABLE 4 | Odds ratios of oocyte yield indices after cumulative embryo transfer according to age groups.

	Logistic regression			Logistic regression age-adjusted		
	OR	**IC95%**	***p***	**OR**	**IC95%**	***p***
Oc/AFC	**1.63**	**[0.85–3.14]**	**0.14**	**1.52**	**[0.77–2.99]**	**0.22**
Age (years)						<0.01
<36				1		
[36–39]				0.49	[0.28–0.86]	
>39				0.22	[0.09–0.52]	
MII/AFC	**2.42**	**[1.14–5.14]**	**0.02**	**2.23**	**[1.02–4.88]**	**0.04**
Age (years)						<0.01
<36				1	–	
[36–39]				0.50	[0.29–0.87]	
>39				0.23	[0.10–0.53]	
Oc/AMH	**0.99**	**[0.94–1.05]**	**0.85**	**1.01**	**[0.96–1.07]**	**0.69**
Age (years)						<0.01
<36				1	–	
[36–39]				0.47	[0.27–0.82]	
>39				0.21	[0.09–0.51]	
MII/AMH	**1.01**	**[0.95–1.08]**	**0.66**	**1.03**	**[0.97–1.10]**	**0.33**
Age (years)						<0.01
<36				1	–	
[36–39]				0.47	[0.27–0.82]	
>39				0.21	[0.09–0.49]	

Oc/AFC, total retrieved oocytes relative to antral follicular count.
MII/AFC, total mature oocytes relative to antral follicular count.
Oc/AMH, total retrieved oocytes relative to serum AMH ng/mL level.
MII/AMH, total mature oocytes relative to serum AMH ng/mL level.

Our data suffer from the weakness of retrospective nature of its design and the lack of homogeneity and AMH dosing techniques making the interpretation of ratios involving it uninterpretable. However, it has the advantage of including a population that reflects our day-to-day practices without excluding PCOS profiles and considering only first attempts in our center, contrary to what was previously done. The outcomes of ART attempts were evaluated up to LB and we were able to compare the cumulative results, which is not often done in previous studies (15, 26).

In conclusion, the search for the ideal index capable of predicting oocyte potential and the chances of obtaining a live birth remains extremely relevant. Our indexes involving serum AMH level have not demonstrated a sufficiently strong prediction. On the other hand, our indices involving AFC (Oc/AFC and MII/AFC) seem promising and could be interesting candidates to validate in a prospective cohort study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MP, RY, AB, and JA contributed to the protocol elaboration, data collection, results interpretation, and manuscript writing and editing. PP and DZ contribute to results analysis and manuscript editing. JT contribute to statistical analysis. All authors contributed to the article and approved the submitted version.

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Coagulation and Fibrinolysis Biomarkers as Potential Indicators for the Diagnosis and Classification of Ovarian Hyperstimulation Syndrome

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Background: Accurate diagnosis and classification of ovarian hyperstimulation syndrome (OHSS) is important for its management. We employed a new high-sensitivity chemiluminescence immunoassay to detect the thrombin-antithrombin complex (TAT), plasmin alpha2-plasmin inhibitor complex (PIC), soluble thrombomodulin (sTM), and tissue plasminogen activator-inhibitor complex (TPAI-C), and evaluated their diagnostic and classification performance for OHSS.

Methods: A total of 106 women were enrolled, including 51 patients with OHSS (25 mild or moderate OHSS, 26 severe OHSS), and 55 without OHSS (control group). TAT, PIC, sTM, and TPAI-C levels were measured using the Sysmex HISCL5000 automated analyzer.

Results: Compared to the control group, TAT, PIC, and TPAI-C levels were significantly higher ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively), whereas the sTM level was significantly lower ($P < 0.001$) in the patients with OHSS. The receiver operating characteristic was used to evaluate the diagnostic efficiency. For the diagnosis of OHSS, the area under the curves (AUCs) for TAT, PIC, sTM, and TPAI-C were 0.991, 0.973, 0.809, and 0.722, respectively. In particular, the sensitivity, specificity, positive predictive value, and negative predictive value for TAT and PIC were all above 90%. For the differential diagnosis of mild–moderate and severe OHSS, the AUCs for TAT, PIC, and TPAI-C were 0.736, 0.735, and 0.818, respectively. The cutoff values of TAT, PIC, and TPAI-C for the differential diagnosis of mild–moderate and severe OHSS were 11.5 ng/mL, 2.4 μg/mL, and 5.8 ng/mL, respectively. Based on these cutoff values, eight cases of mild–moderate OHSS exceeded the cutoff values, two of which developed to severe OHSS in the following days. However, of the remaining 17 cases of mild–moderate OHSS patients with negative biomarkers, none subsequently developed severe OHSS.

Conclusions: TAT, PIC, sTM, and TPAI-C can be used as sensitive biomarkers in the diagnosis of OHSS. Meanwhile, TAT, PIC, and TPAI-C also displayed remarkable potential in the classification of OHSS. In addition, the levels of TAT, PIC, and TPAI-C above the cutoff values in patients with mild–moderate OHSS might predict a high risk of developing severe OHSS.

Keywords: ovarian hyperstimulation syndrome, thrombin-antithrombin complex, plasmin alpha2-plasmin inhibitor complex, soluble thrombomodulin, tissue plasminogen activator-inhibitor complex, receiver operating characteristic

INTRODUCTION

Ovarian hyperstimulation syndrome (OHSS) is a major iatrogenic complication associated with controlled ovarian stimulation during *in vitro* fertilization (IVF). The reported incidence of OHSS varies markedly, and is estimated to be 0.5–5%, and even up to 10% in high-risk women (1–4). However, the true incidence of OHSS is difficult to estimate because of the lack of strict universally accepted diagnostic criteria. The precise pathogenesis of OHSS is unclear, but is believed to involve pro-inflammatory mediators produced by the use of human chorion gonadotrophin (hCG) for the triggering of final oocyte maturation (5). Women with OHSS demonstrate ovarian enlargement and increased vascular permeability. There is a shift of fluids from the intravascular compartment into the third space, mediated by the elevated levels of vascular endothelial growth factor (VEGF) secreted by the granulosa lutein cells (6).

Hypercoagulability is a common syndrome in patients with OHSS. Moreover, if hypercoagulability develops further, thrombosis may occur, which is the most serious and life-threatening complication of IVF. Thromboembolic disease has been reported in many sites of patients with OHSS, including in the internal jugular, subclavian, axillary, ulnar, popliteal, cortical, mesenteric, coronary, and cerebral vessels (7–9). To prevent the occurrence of thrombosis, it is crucial to accurately assess the hypercoagulability in patients with OHSS.

However, according to the existing diagnostic and classification criteria of OHSS, such as the Golan criteria, the guidelines published by the Practice Committee of the American Society for Reproductive Medicine and the Royal College of Obstetricians & Gynecologists, no hemostasis indicators are available (10–12). Currently, white blood cell (WBC) count and hematocrit (Hct) are the two most commonly used laboratory indicators. Unfortunately, an elevated WBC count may be secondary to a generalized stress reaction and hemoconcentration, and Hct mainly reflects intravascular volume depletion and blood viscosity (13). Neither can be used to reflect the hemostatic system. The absence of hemostasis indicators has resulted in a lack of laboratory guidance in the diagnosis and subsequent anticoagulant therapy of OHSS. Considering that conventional coagulation tests, such as activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT), are not suitable for assessing the hypercoagulability, novel coagulation and fibrinolysis biomarkers need to be explored.

The thrombin-antithrombin complex (TAT), plasmin alpha2-plasmin inhibitor complex (PIC), soluble thrombomodulin (sTM), and tissue plasminogen activator (t-PA)-inhibitor complex (TPAI-C) are four sensitive coagulation and fibrinolysis biomarkers in the hemostatic system. Elevated levels of these have been found in many underlying diseases. TAT, PIC, sTM, and TPAI-C levels are significantly higher in patients with disseminated intravascular coagulation (DIC) than in those with non-overt DIC, and TAT, sTM, and TPAI-C levels are significantly higher in patients with pre-DIC than in those with non-overt DIC (14). Yuying Cheng et al. confirmed the clinical value of the four biomarkers in predicting postoperative venous thromboembolism in total joint arthroplasty patients (15). In addition, the diagnostic and prognostic values of these biomarkers have also been demonstrated in patients with malignant tumors with venous thrombosis (16, 17).

In this study, we aimed to evaluate the diagnostic and classification value of TAT, PIC, sTM, and TPAI-C for OHSS, and preliminarily evaluate the potential use of these biomarkers in clinical practice in a Chinese population.

MATERIALS AND METHODS

Participants

This study was performed at the Women's Hospital, School of Medicine, Zhejiang University (China) from July 2020 to February 2021. The study protocol was approved by the ethics committee of the hospital (approval number: IRB-20200133-R). Eligible participants were those who underwent IVF using either the gonadotropin-releasing hormone agonist (GnRHa) long protocol or the GnRH antagonist protocol and suffered from OHSS. The exclusion criteria were data missing from the database; age over 45 years; receiving GnRH agonist trigger or withholding hCG; any known hereditary or acquired thrombotic or bleeding disorder; having already received anticoagulant therapy; and chronic diseases, such as cardiovascular disease or diabetes mellitus.

During this period, 83 patients were diagnosed with OHSS, 32 of which were excluded based on the exclusion criteria, and 51 were enrolled in the final study, including 25 patients with mild or moderate OHSS and 26 with severe OHSS, according to Golan and Wasserman's 2009 criteria (10). All patients with OHSS were diagnosed within 9 days after oocyte retrieval. The control group consisted of 55 participants who underwent IVF using either the GnRHa long protocol or the GnRH antagonist protocol and did

not develop OHSS during the same time period. Women in the control group were followed up throughout the first trimester to ensure that OHSS did not occur. The information collected for each participant was age; body mass index (BMI); IVF protocol; gonadotropin doses; antral follicle count; basal levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, progesterone, and anti-Müllerian hormone (AMH); levels of estradiol on the day of hCG administration; levels of progesterone on the day of oocyte retrieval; and the number of oocytes retrieved.

Controlled Ovarian Hyperstimulation Protocol

The standard treatment protocol for controlled ovarian hyperstimulation (COH) using the GnRHa long protocol or GnRH antagonist protocol was applied to all participants. In the GnRHa long protocol, GnRHa was administered in the midluteal phase preceding the cycle to downregulate estrogen production. Thereafter, COH was performed by the administration of both recombinant FSH (rFSH) and human menopausal gonadotropin (HMG), depending on age, BMI, antral follicle count, size and number of follicles, and estradiol levels. The initial doses of rFSH ranged from 150–225 IU/day, and those of HMG ranged from 75–150 IU/day. The dose was subsequently adjusted depending on the ovarian response, as evaluated by the E2 levels and combined with ultrasound. When at least three follicles reached a mean diameter of 17 mm, 6,500 IU of hCG was injected. The transvaginal ultrasound-guided oocyte retrieval was performed ∼36 h after hCG injection.

In the GnRH antagonist protocol, rFSH or HMG was injected from the third day of the last menstrual period. The initial doses of rFSH and HMG were the same as those for the GnRHa long protocol, and the dose was subsequently adjusted, depending on the ovarian response. When the dominant follicle diameter was ∼12–14 mm, the GnRH antagonist was administered until the day of hCG administration. When at least three follicles reached a mean diameter of 17 mm, 6,500 IU of hCG was injected, and oocyte retrieval was scheduled ∼36 h after hCG injection.

Blood Samples and Laboratory Assays

Blood samples were obtained immediately after OHSS was diagnosed and before any heparin treatment. For the control group, blood samples were obtained ∼2 weeks after embryo transfer when the patient visited the hospital for the hCG test. If the hCG level was above 5.3 IU/L (reference interval: <5.3 IU/L), the participant would be followed up throughout the first trimester to ensure that OHSS did not occur, as previously described.

Blood samples were collected in a vacuum tube containing 0.109 M of dehydrated sodium citrate (BD Vacutainer Systems), centrifuged at 2,500 × *g* for 10 min, and then PT, APTT, thrombin time (TT), and fibrinogen concentration were measured by the clotting method on the STA-R MAX Coagulation Analyzer (Diagnostica Stago). The rest of the plasma samples were stored at −80°C for the assay of TAT, PIC, sTM, and TPAI-C. The four biomarkers were measured using the HISCL 5000 Automatic Chemiluminescence Immunoanalyzer (Sysmex Corporation) within 6 months. In addition, blood samples were collected in a vacuum tube containing EDTA-K2 (BD Vacutainer Systems) for the measurement of WBCs and Hct. WBC counts and Hct were determined on the Sysmex XN9000 Automatic Hematology Analyzer (Sysmex Corporation). All tests were performed with the original reagents and undertaken according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using the SPSS 20.0 software. A P-value < 0.05 was considered significant for all comparisons. The Shapiro-Wilk test was used to verify normality. Descriptive statistics are presented as mean ± SD for normally distributed variables and median (25th−75th percentile) for non-normally distributed variables. Parametric (t-test) and non-parametric (Mann-Whitney U test) analyses were performed for normally and non-normally distributed variables, respectively. Spearman's rank correlation analysis was used to analyze the association between the levels of these biomarkers and those of WBCs and Hct. Receiver operating characteristic (ROC) curves analysis was used to evaluate the diagnostic efficiency, and the maximum value of the Youden index served as the cutoff value. Sensitivities, specificities, positive predictive values (PPVs), and negative predictive values (NPVs) were calculated using standard formulas and were expressed as percentages [95% confidence interval (CI)].

RESULTS

Baseline Clinical Characteristics of the Participants

Overall, 51 female patients with OHSS were enrolled in this study, including 25 with mild or moderate OHSS and 26 with severe OHSS. The control group consisted of 55 participants undergoing IVF who did not develop OHSS during the same time period. The baseline clinical characteristics of these participants are shown in **Table 1**. As expected, no significant differences were found in the conventional coagulation tests (PT, APTT, and TT) ($P = 0.486$, $P = 0.286$, $P = 0.805$, respectively). The levels of fibrinogen, WBCs, and Hct were significantly higher in the OHSS group than the control group ($P < 0.001$, $P < 0.001$, $P = 0.001$, respectively). The levels of TAT, PIC, sTM, and TPAI-C did not demonstrate any significant differences between the two therapeutic protocols ($P = 0.946$, $P = 0.872$, $P = 0.467$, $P = 0.268$, respectively).

Comparison of TAT, PIC, sTM, and TPAI-C Levels in Patients With OHSS and Control Patients

As shown in **Figure 1**, compared to the control group, TAT, PIC, and TPAI-C levels were significantly higher ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) and the sTM level was significantly lower ($P < 0.001$) in the OHSS group. The median plasma levels of TAT, PIC, sTM, and TPAI-C (OHSS vs. control group) were 7.4 (2.7–11.6) vs. 0.5 (0.4–0.6) ng/mL, 1.8 (1.2–2.8) vs. 0.4 (0.3–0.5) μg/mL, 5.6 (4.9–6.6) vs. 7.0 (6.4–7.5) TU/mL, and 5.1 (2.9–7.6) vs. 3.5 (2.3–4.6) ng/mL, respectively.

TABLE 1 | Baseline clinical characteristics of the participants.

	OHSS ($n = 51$)	Controls ($n = 55$)	*P*
Age (years)	30.3 ± 3.7	32.7 ± 4.8	0.007
BMI (kg/m^2)	22.1 ± 2.7	21.9 ± 2.5	0.805
Antral follicle count	13.0 ± 5.9	11.6 ± 4.3	0.159
AMH (ng/mL)	7.02 (4.80–10.72)	2.37 (1.26–4.45)	0.000
Basal LH (IU/L)	5.93 (3.76–8.36)	4.81 (3.02–6.45)	0.093
Basal FSH (IU/L)	5.44 (4.64–6.24)	6.06 (4.80–7.55)	0.127
Basal estradiol (pmol/L)	129.99 ± 50.06	142.03 ± 72.51	0.320
Basal progesterone (nmol/L)	1.12 (0.63–1.49)	0.96 (0.77–1.26)	0.144
Estradiol on the day of hCG administration (pmol/L)	19,258.00 (12,155.00–31,990.00)	8,344.00 (4,445.00–11,404.00)	0.000
Progesterone on the oocyte retrieval day (nmol/L)	21.45 (13.33–43.02)	11.08 (8.36–17.13)	0.000
IVF protocol [*n* (%)]			
Long protocol	31 (60.7)	32 (58.2)	0.844
Antagonist protocol	20 (39.3)	23 (41.8)	0.844
Number of oocytes retrieved	18.0 ± 10.1	10.2 ± 6.4	0.000
Conventional coagulation tests			
PT (s)	12.9 ± 0.5	13.0 ± 0.6	0.486
APTT (s)	33.9 ± 3.6	34.6 ± 3.1	0.286
TT (s)	15.6 ± 1.1	15.7 ± 0.8	0.805
Fibrinogen (g/L)	5.04 ± 1.53	3.20 ± 0.57	0.000
Blood routine examination			
WBC (×109/L)	11.8 (9.2–14.9)	7.1 (6.4–9.6)	0.000
Hct (%)	41.8 ± 4.9	39.0 ± 2.5	0.001

Data are presented as mean ± SD or median (25th−75th percentile) or No (%). OHSS, ovarian hyperstimulation syndrome; BMI, body mass index; AMH, anti-Müllerian hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; IVF, in vitro fertilization; Gn, gonadotropin; PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; WBC, white blood cell; Hct, hematocrit.

Evaluation of the Correlations Between TAT, PIC, sTM, and TPAI-C Levels and WBC and Hct Levels

As WBC and Hct levels are two routine laboratory indicators in the diagnosis and classification of OHSS, we investigated the correlations between TAT, PIC, sTM, and TPAI-C levels and WBC and Hct levels. As shown in **Figure 2**, TAT, PIC, and TPAI-C levels were positively correlated with WBC levels ($r = 0.531$, $P < 0.001$; $r = 0.646$, $P < 0.001$; $r = 0.471$, $P < 0.001$, respectively). However, the sTM level was negatively correlated with the WBC level ($r = -0.210$, $P < 0.05$). TAT, PIC, and TPAI-C levels were also positively correlated with the Hct level ($r = 0.247$, $P < 0.05$; $r = 0.323$, $P < 0.001$; $r = 0.411$, $P < 0.001$, respectively). There was no significant correlation between sTM and Hct ($r = -0.135$, $P = 0.169$). Based on a comparison, the correlations between the biomarker and WBC levels were higher than those between the biomarker and Hct levels. The correlations between sTM and WBC/Hct levels were lower than those between TAT/PIC/TPAI-C and WBC/Hct levels.

Diagnostic Efficiency of TAT, PIC, sTM, and TPAI-C for OHSS

Given the marked changes in TAT, PIC, sTM, and TPAI-C levels in patients with OHSS, we further evaluated them as potential biomarkers for the diagnosis of OHSS using ROC curve analysis. Among the four biomarkers, the highest area under the curve (AUC) was observed for TAT and the lowest was observed for TPAI-C (**Figure 3A**). The optimal cutoff values were defined as the sum of maximum sensitivity and specificity. As shown in **Table 2**, the AUCs for TAT and PIC were 0.991 (95% CI, 0.980–1.000) and 0.973 (95% CI, 0.944–1.000), respectively, which were higher than the values for sTM and TPAI-C; namely, 0.809 (95% CI, 0.725–0.892) and 0.722 (95% CI, 0.622–0.820), respectively. In particular, the sensitivity, specificity, PPV, and NPV for TAT and PIC were all above 90%, which showed a good diagnostic value for OHSS. The specificities for sTM and TPAI-C were 94.5 and 83.6%, with a sensitivity of 54.9 and 56.9%, respectively. According to the ROC curve, the cutoff values of TAT, PIC, sTM, and TPAI-C for the diagnosis of OHSS were 1.2 ng/mL, 0.7 μg/mL, 5.7 TU/mL, and 5.0 ng/mL, respectively.

Differential Diagnostic Efficiency of TAT, PIC, sTM, and TPAI-C for Mild–Moderate and Severe OHSS

Compared to patients with mild–moderate OHSS, TAT, PIC, and TPAI-C levels were significantly higher in those with severe OHSS ($P = 0.004$, $P = 0.004$, $P < 0.001$, respectively), whereas the sTM level was not significantly different between the two groups ($P = 0.940$). We further investigated the differential diagnostic value of these biomarkers for mild–moderate and severe OHSS using ROC curve analysis (**Figure 3B**). As shown in **Table 2**, the AUCs for TAT, PIC, and TPAI-C in the differential diagnosis of mild–moderate and severe OHSS were 0.736, 0.735, and 0.818, respectively. However, the AUC for sTM was merely 0.506, which suggested that it cannot be used for the differential diagnosis of mild–moderate or severe OHSS. The cutoff values of TAT, PIC, and TPAI-C for the differential diagnosis of mild–moderate and severe OHSS were 11.5 ng/mL, 2.4 μg/mL, and 5.8 ng/mL, respectively. TPAI-C showed the highest differential diagnosis value, with a sensitivity of 73.1% and a specificity of 88%.

Evaluations of the Outcomes of Patients With Mild–Moderate OHSS With Positive Biomarkers

The cutoff values of TAT, PIC, and TPAI-C for the differential diagnosis of mild–moderate and severe OHSS were 11.5 ng/mL, 2.4 μg/mL, and 5.8 ng/mL, respectively. Of the 25 patients with mild–moderate OHSS, eight had a positive result for at least one biomarker (above the cutoff values), and 17 had a

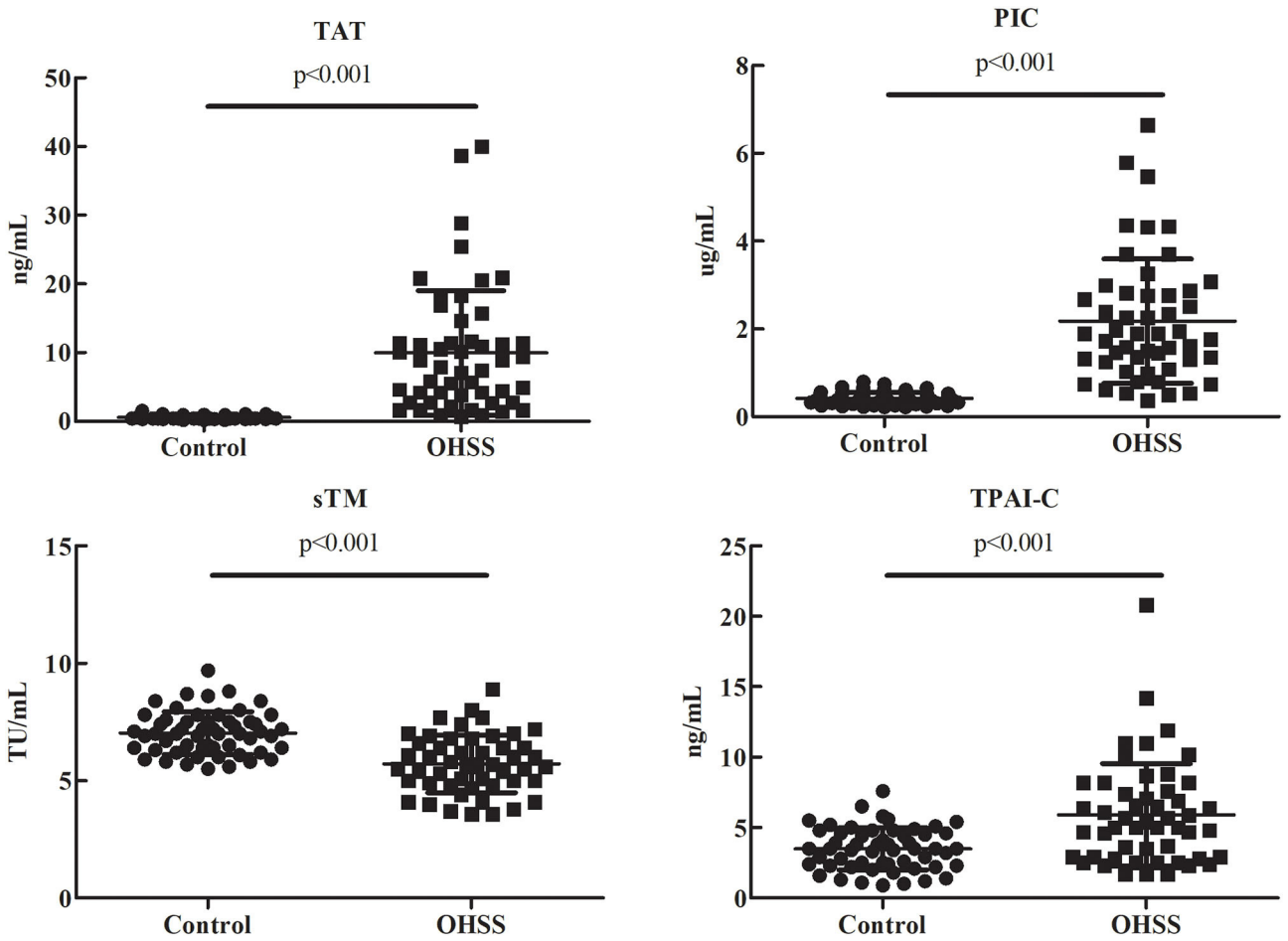

FIGURE 1 | Comparisons of the thrombin-antithrombin complex, plasmin alpha2-plasmin inhibitor complex, soluble thrombomodulin, and tissue plasminogen activator-inhibitor complex levels in patients with ovarian hyperstimulation syndrome and control patients. TAT, thrombin-antithrombin complex; PIC, plasmin alpha2-plasmin inhibitor complex; sTM, soluble thrombomodulin; TPAI-C, tissue plasminogen activator-inhibitor complex; OHSS, ovarian hyperstimulation syndrome.

negative result for all biomarkers (below the cutoff values). The management measures for these patients in clinical practice involved increasing fluid intake, supportive care, and intensive monitoring (blood pressure, pulse, daily weight, daily urine volume, laboratory parameters, etc.). Subsequently, we followed up these patients and found that two of the eight patients with positive biomarkers developed severe OHSS in the following days, and none of the 17 patients with negative biomarkers developed severe OHSS (**Table 3**).

DISCUSSION

Accurate diagnosis and classification of OHSS is important for the identification of suitable treatments. However, although a number of diagnostic and classification systems of OHSS have been developed, there are currently no universally agreed upon criteria (10, 11). Furthermore, current diagnostic and classification systems inadequately capture OHSS in clinical practice in a uniform manner, as no hemostatic indicators are involved. Hence, there is an urgent need to explore novel coagulation and fibrinolysis biomarkers for OHSS, despite considerable challenges.

In this study, we first investigated the levels of the conventional coagulation tests in patients with OHSS. No significant differences were found in the levels of PT, APTT, and TT between patients with OHSS and control patients. This might be because these tests are designed primarily to screen for coagulation factor deficiencies, rather than hypercoagulability, which is usually related to excessive coagulation factors (18, 19). Although fibrinogen levels were significantly higher in patients with OHSS vs. control patients, however, given that fibrinogen was an indicator of acute phase of reaction, and the increase of fibrinogen levels came not only from disorders of the hemostasis system, but also from inflammation and other stress responses, fibrinogen was not a specific indicator for the diagnosis and classification of OHSS.

Our results indicated that TAT, PIC, sTM, and TPAI-C had good diagnostic performance for OHSS; meanwhile, we showed that TAT, PIC, and TPAI-C can also be used to classify the severity of OHSS. TAT is a molecular complex composed of

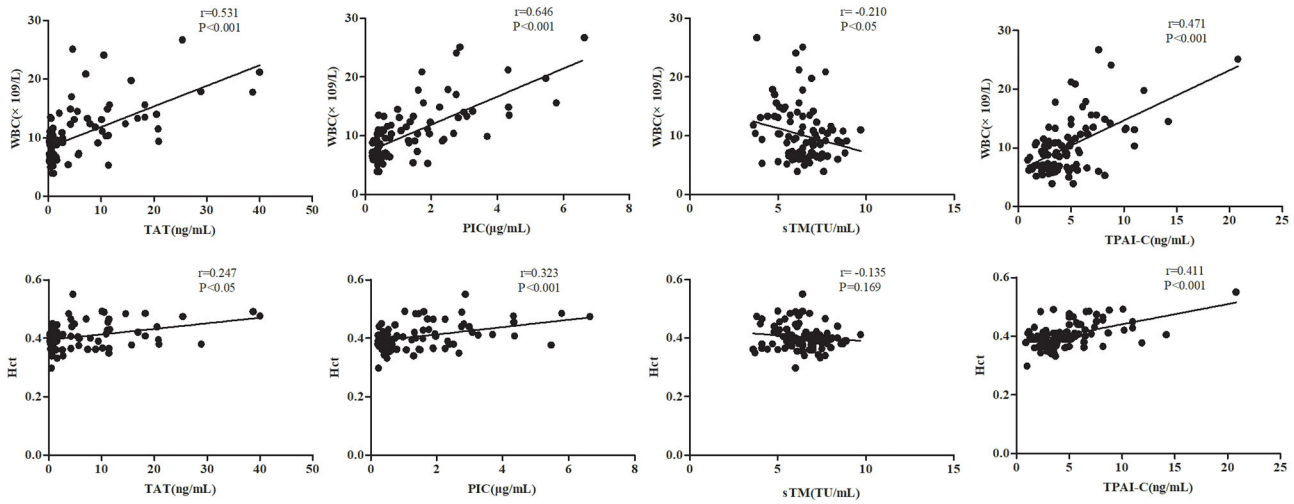

FIGURE 2 | Correlations between the thrombin-antithrombin complex, plasmin alpha2-plasmin inhibitor complex, soluble thrombomodulin, and tissue plasminogen activator-inhibitor complex levels and white blood cell and hematocrit levels. TAT, thrombin-antithrombin complex; PIC, plasmin alpha2-plasmin inhibitor complex; sTM, soluble thrombomodulin; TPAI-C, tissue plasminogen activator-inhibitor complex; WBC, white blood cell; Hct, hematocrit.

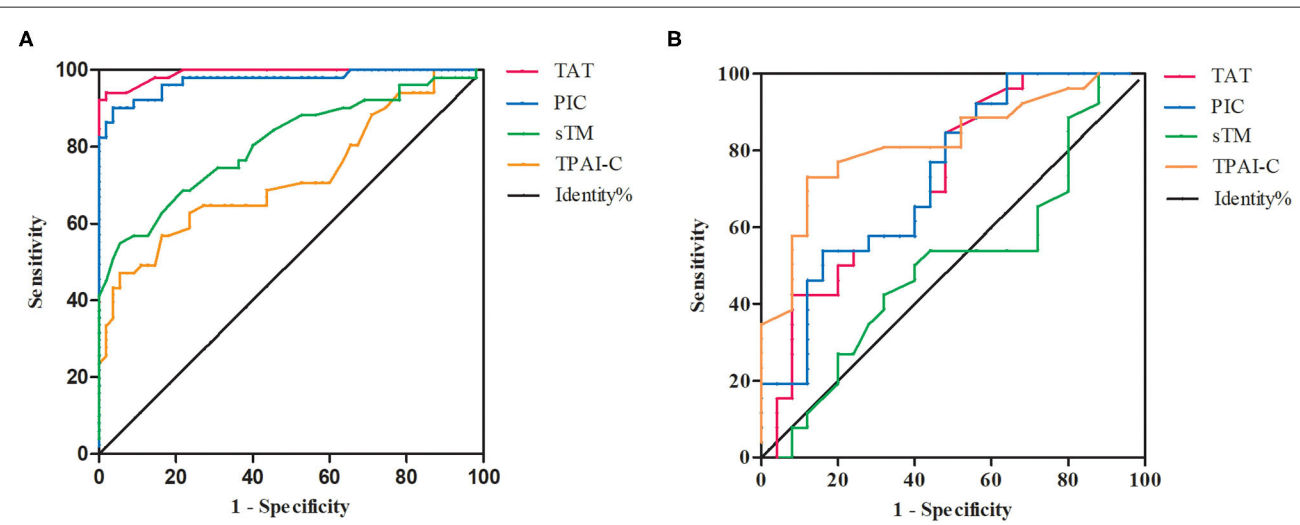

FIGURE 3 | The receiver operating characteristic (ROC) curves of thrombin-antithrombin complex, plasmin alpha2-plasmin inhibitor complex, soluble thrombomodulin, and tissue plasminogen activator-inhibitor complex. **(A)** The ROC curves for the diagnosis of ovarian hyperstimulation syndrome (OHSS). **(B)** The ROC curves for the differential diagnosis of mild–moderate and severe OHSS. TAT, thrombin-antithrombin complex; PIC, plasmin alpha2-plasmin inhibitor complex; sTM, soluble thrombomodulin; TPAI-C, tissue plasminogen activator-inhibitor complex.

thrombin and antithrombin and is considered to be a sensitive marker of thrombin formation and coagulation activation. PIC is a molecular complex composed of plasmin and alpha2-plasmin inhibitor, and is a marker of plasmin formation and fibrinolysis activation. sTM is not only an indicator of endothelial injury, but can also be combined with thrombin to play an anticoagulant role. TPAI-C is formed through the combination of t-PA and plasminogen activator inhibitor 1, and is a marker of endothelial injury and fibrinolysis activation. Therefore, these biomarkers are highly sensitive coagulation and fibrinolysis indicators and may highlight even minimal hemostatic activation (14). The data of the present study also supported this. We found that TAT, PIC, and TPAI-C levels were significantly higher, whereas sTM levels were significantly lower, in patients with OHSS vs. control patients. This suggested that there was an imbalance between coagulation and fibrinolysis in patients with OHSS. Although the exact mechanism has not yet been fully elucidated, the imbalance may play a vital role in the thrombosis of patients with OHSS. These results are in accordance with those of several previous studies (20, 21). In addition, in contrast to the other biomarkers, sTM levels in patients with OHSS were significantly lower than those in control subjects. As sTM is not only an indicator of

TABLE 2 | Evaluation of thrombin-antithrombin complex, plasmin alpha2-plasmin inhibitor complex, soluble thrombomodulin, and tissue plasminogen activator-inhibitor complex efficiency [including the diagnostic efficiency for ovarian hyperstimulation syndrome (OHSS) and the differential diagnostic efficiency for mild–moderate and severe OHSS].

	Diagnostic efficiency for OHSS				Differential diagnostic efficiency for severe OHSS			
	TAT	**PIC**	**sTM**	**TPAI-C**	**TAT**	**PIC**	**sTM**	**TPAI-C**
AUC	0.991	0.973	0.809	0.722	0.736	0.735	0.506	0.818
95%CI	0.980–1.000	0.944–1.000	0.725–0.892	0.622–0.820	0.600–0.872	0.598–0.872	0.344–0.669	0.699–0.936
Cutoff value	1.2	0.7	5.7	5.0	11.5	2.4	6.1	5.8
Sensitivity (%)	94.1	90.2	54.9	56.9	42.3	53.8	42.3	73.1
Specificity (%)	98.2	96.4	94.5	83.6	92	84	68	88
PPV	97.9	95.8	90.6	76.3	84.6	76.5	48.9	86.4
NPV	94.7	91.3	69.3	68.7	68.4	61.8	44.4	75.9
Youden's index	0.923	0.865	0.494	0.405	0.343	0.378	0.103	0.611

OHSS, ovarian hyperstimulation syndrome; TAT, thrombin-antithrombin complex; PIC, plasmin alpha2-plasmin inhibitor complex; sTM, soluble thrombomodulin; TPAI-C, tissue plasminogen activator-inhibitor complex; AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

TABLE 3 | The outcomes of patients with mild–moderate ovarian hyperstimulation syndrome with positive biomarkers.

	Clinical diagnosis	**TAT, ng/mL**	**PIC, μg/mL**	**TPAI-C, ng/mL**	**Clinical outcomes**
Case 1	Mild OHSS	20.8*	1.2	2.4	Improved
Case 2	Moderate OHSS	20.0*	4.3*	5.0	Developed severe OHSS
Case 3	Mild OHSS	11.4	2.7*	2.9	Improved
Case 4	Moderate OHSS	2.7	3.7*	2.5	Improved
Case 5	Mild OHSS	2.6	3.5*	2.4	Improved
Case 6	Mild OHSS	6.9	1.4	6.5*	Improved
Case 7	Moderate OHSS	11.4	1.7	8.2*	Developed severe OHSS
Case 8	Mild OHSS	11.1	1.9	8.4*	Improved

**Positive biomarkers: TAT > 11.5 ng/mL, PIC > 2.4 μg/mL, TPAI-C > 5.8 ng/mL. OHSS, ovarian hyperstimulation syndrome; TAT, thrombin-antithrombin complex; PIC, plasmin alpha2-plasmin inhibitor complex; TPAI-C, tissue plasminogen activator-inhibitor complex.*

endothelial injury, but also combines with thrombin to play an anticoagulant role, this decrease in levels may contribute to the tendency of thrombosis development in patients with OHSS.

As WBC and Hct levels are the two most commonly used indicators in the existing diagnostic and classification criteria of OHSS, we investigated the correlations between biomarker levels and WBC and Hct levels. Except for sTM, significant correlations were observed between the biomarker levels and WBC and Hct levels. The highest correlation was observed between PIC and WBC levels ($r = 0.646$, $P < 0.001$). Owing to the significant correlations between the biomarkers and WBC levels, Hct levels further confirmed their diagnostic and classification value. The correlations between the biomarker and Hct levels were relatively weak. As Hct mainly reflects hemoconcentration, the relatively weak correlation implies that other mechanisms contribute to the hypercoagulability in OHSS other than hemoconcentration. This is consistent with the results of a study by Zohav et al. (22).

The AUCs for the diagnostic biomarkers TAT, PIC, sTM, and TPAI-C were 0.991, 0.973, 0.809, and 0.722, respectively. These biomarkers showed good diagnostic efficiency for OHSS. In particular, the sensitivity, specificity, PPV, and NPV for TAT and PIC were all above 90%, which showed extremely high diagnostic value. Once a diagnosis of OHSS is made, disease severity should be classified. The treatment approach for the clinical management of OHSS is multifaceted and individualized based on disease severity and progression. Most mild and moderate cases of OHSS are self-limited and require only intensive monitoring in the outpatient department; however, severe OHSS requires hospitalization to relieve symptoms and control the progression of the disease (23, 24). In this study, TAT, PIC, and sTM levels in the mild–moderate OHSS group were significantly higher than those in the control group ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). Meanwhile, TAT, PIC, and TPAI-C levels in the severe OHSS group were significantly higher than those in the mild–moderate OHSS group ($P = 0.004$, $P = 0.004$, and $P < 0.001$, respectively). This revealed that the hypercoagulability in OHSS was a gradual process. Therefore, these biomarkers might have important roles in its classification. The ROC curve analysis results of the present study also supported this. We investigated the differential diagnostic value of these biomarkers for mild–moderate and severe OHSS. Except for sTM, all biomarkers showed significant potential value for the classification of OHSS. The highest AUC was observed for TPAI-C (0.818), with a sensitivity of 73.1% and a specificity of 88%, which suggested that TPAI-C was the most optimal biomarker. The cutoff value

for TPAI-C in the differential diagnosis for mild–moderate and severe OHSS was 5.8 ng/mL.

In this study, to further validate the value of these biomarkers in clinical practice, we followed up the outcomes of the patients with mild–moderate OHSS. There were eight patients with mild–moderate OHSS who exceeded the cutoff values obtained by ROC analysis, two of which developed severe OHSS in the following days. However, of the remaining 17 patients with mild–moderate OHSS with negative biomarkers (below the cutoff values), none subsequently developed severe OHSS. These results indicated that the patients with mild–moderate OHSS with positive biomarkers were at a high risk of developing severe OHSS; namely, the presence of positive biomarkers in patients with mild–moderate OHSS might predict a poor prognosis. Considering that the treatment approach for OHSS is multifaceted and individualized based on disease severity and progression, these biomarkers could help identify high risk patients with mild–moderate OHSS who need to be more closely monitored and who may even require early prophylactic anticoagulant therapy.

In addition, the clinical application of these biomarkers has been markedly restricted owing to the inconvenient and inefficient detection methodology used in the past. Currently, with the development of the high-sensitivity chemiluminescence immunoassay, these biomarkers can be detected quickly and automatically in a clinical laboratory. For the test used in this study, the minimum volume for a sample was 20 μL, and the results were available within 17 min. The improvement of the methodology will facilitate the spread of these biomarkers to thousands of laboratories in China.

There were some limitations in this study. The sample size was not sufficiently large, especially after grouping. Therefore, larger sample studies are needed. Another limitation is that all participants were subjected to a single sampling. If a series of sampling is performed at different time points, such as during COH, on the day of hCG administration, and on the oocyte retrieval day, this will allow the evaluation of the value of these biomarkers more comprehensively, which may be the focus of our future studies.

In conclusion, we found that TAT, PIC, sTM, and TPAI-C can be used as sensitive biomarkers in the diagnosis of OHSS. Meanwhile, TAT, PIC, and TPAI-C also displayed remarkable potential in the classification of OHSS. In addition, the levels of TAT, PIC, and TPAI-C above the cutoff values in patients with mild–moderate OHSS might predict a high risk of developing severe OHSS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the Women's Hospital, School of Medicine, Zhejiang University (China). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SLi, YQ, and SLu conceived the idea and designed the study and drafted the article. SLu revised the article. YP and KW contributed the collection and analysis of the data. All authors have approved the submitted version.

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Comparison of Hysterosalpingography With Laparoscopy in the Diagnosis of Tubal Factor of Female Infertility

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Background: Laparoscopy is considered to be the gold standard in the evaluation of causes leading to infertility. Hysterosalpingography (HSG) permits indirect visualization of the cervical canal, uterine cavity, and tube patency, which is helpful for evaluating the causes of infertility.

Objective: This study aimed to detect tubal abnormalities in infertile women by HSG or laparoscopy and determine the value of HSG in diagnosing fallopian tube status.

Methods: The study group consisted of 1,276 patients. HSG was performed as a preliminary test for the evaluation of fallopian tube status. Women were subjected to laparoscopic examination on evidence of HSG abnormalities.

Results: The negative predictive value of HSG for detecting patency or occlusion for the right/left tube was 92.08 and 95.44%, respectively. The kappa values for the consistent diagnosis in the right/left tube were 0.470 and 0.574, respectively. In cases of low patency of the right/left tube, there was a greater than a 40% chance for the tube to be patent, and the remaining high probability was pelvic adhesion. The positive predictive value of HSG for detecting patency or occlusion for both tubes was 87.2%. The kappa value was 0.898 [95% CI (0.838, 0.937), $p < 0.001$], which meant that the diagnostic accuracy of HSG for both tube patency/occlusion was explicit. The kappa value for the diagnosis of hydrosalpinx (especially for bilateral tube hydrosalpinx) was 0.838 [95% CI (0.754, 0.922), $p < 0.001$], and the diagnostic accuracy for HSG was 79.8, 67.9, and 72.4%, respectively.

Conclusion: The current study concluded that HSG is a good diagnostic modality to detect tube abnormalities in infertile patients. HSG and laparoscopy are complementary to each other and whenever the patient is undertaken for diagnosis of infertility. Cost-effective HSG had good predictive value in identifying tubal factor infertility.

Keywords: hysterosalpingography, laparoscopy, infertility, diagnose, fallopian tubes

INTRODUCTION

Infertility is defined as a failure of conception in a couple who has a regular unprotected sexual activity for 1 year and still does not conceive (1). Many factors can result in infertility, including disorder in fallopian tubes, anovulation, and pelvic adhesion leading to pelvic microenvironments. Among the factors mentioned above, disorders of the fallopian tube account for 30–45% of the reasons for infertility (2, 3). Hence, screening for tubal occlusion is one of the first essential steps of infertility assessment. In recent years, with the development of endoscopic techniques, the diagnosis and treatment of female infertility have made significant advances (3, 4).

Hysterosalpingography (HSG) is a contrast-enhanced fluoroscopic radiological technique adopted to evaluate the uterine cavity, fallopian tubes, and adjacent peritoneum after injection of contrast media through the cervical canal (5). It determines the patency of fallopian tubes, the contour of the uterus, and the adjacent pelvic peritoneum in patients experiencing assessment for infertility. Sometimes, HSG gives us the first indication for the underlying reasons leading to infertility (6, 7).

Although HSG provides us with a permanent record of the fluoroscopic examination of the uterine cavity and tubal patency, subtle changes such as pelvic adhesions and endometriosis, which influence fertility without any pelvic anatomy changes, can be missed (7–9). Laparoscopy can magnify some subtle differences in the fallopian tube or pelvic peritoneum. Although it was considered the "gold standard" procedure for determining the reasons for infertility (10–12), it was not recommended as the first-line clinical evaluation test because it is an invasive procedure also needing anesthesia, thus adding to the cost and side effects.

This study aimed to compare the diagnostic value of HSG in evaluating tubal patency and pelvic adhesion in the hope of providing some clinical value in the diagnosis of infertility.

MATERIALS AND METHODS

Patients

From January 2014 to November 2020, we retrospectively studied 1,276 patients who underwent HSG or laparoscopic examination for infertility. First, HSG was performed. If the results of HSG were normal or not patent, but the patients did not become pregnant in the 12 months after examination, we performed a laparoscopic procedure. If the results of HSG were occlusion or hydrosalpinx, but the patients desired to conceive, naturally, they chose to perform the laparoscopic examination. All the enrolled patients had a regular menstrual cycle, and routine semen examination of the husband was normal. We excluded patients who had an ovarian cyst, uterine malformation, endometriosis, or any other type of organic lesion that could be found by routine ultrasonography. The medical ethics committee of the First Affiliated Hospital of Sun Yat-sen University approved the study.

HSG Examination

Hysterosalpingography examination was performed 3–7 days after menstruation. An experienced technician performed the procedures, and two separate radiologists determined the results. According to the image, the patency of the tube could be divided into no patency, patency, and occlusion (**Figure 1**). If combined with hydrosalpinx (**Figure 2**), the diagnosis would then be added. The criteria for low patency were the following: the iodine agent in the whole oviduct was absorbent, but the lumen wall was rough, thickened, narrow, and knotted, or the iodine agent remained for 24 h.

Laparoscopic Examination

The laparoscopic examination was performed 7–12 days after menstruation. The patients underwent this procedure with general anesthesia. During the process, we directly found pelvic adhesion, and methylene blue staining was used to determine the patency of the fallopian tube. If there was no pelvic adhesion and both fallopian tubes were patent, the diagnosis was standard. Otherwise, it was described as pelvic adhesion or occlusion.

Statistical Analysis

We used IBM SPSS Statistics for Windows, Version 26.0. (Armonk, NY: IBM Corp) to conduct statistical analysis. Chi-square or Fisher's exact probability method was used to compare differences between groups. $P < 0.05$ was considered statistically significant. Compared with laparoscopy, which was regarded as the "gold standard" procedure for follicular tube examination, the sensitivity, specificity, and positive and negative predictive values of HSG were calculated. Cohen's kappa coefficient analysis was used to evaluate the consistency of the research methodology.

RESULTS

The General Characteristic of Patients

A total of 1,276 women with a history of infertility who underwent HSG and laparoscopy were included in this study. The mean age of the patients was 30.67 ± 4.92 ($M \pm SD$) years (ranging from 18 to 45 years), and the average number of years of infertility was 2.96 ± 2.08 ($M \pm SD$) years (ranging from 0 to 14 years). Secondary infertility was more frequent ($n = 863$, 67.61%) than primary infertility ($n = 413$, 32.39%), and 20.97% ($n = 181$) of patients had a history of previous pelvic surgery.

The Comparison of HSG and Laparoscopy in the Diagnosis of Unilateral Fallopian Tube Patency or Occlusion

The HSG and laparoscopic diagnosis results of fallopian tubes are shown in **Table 1**. When patients underwent HSG, we tended to diagnose right/left side of fallopian tube non-patency. Compared with the right/left tube patency group, the diagnosis of patency/occlusion/pelvic adhesion in the corresponding right/left tube low patency by laparoscopy was significantly different with p-values < at 0.007 and <0.001, respectively. Further analysis showed that the diagnosis of occlusion tended to increase by laparoscopy in the right/left tube low patency group by HSG with the rate of 11.2 and 22.8%, respectively. In

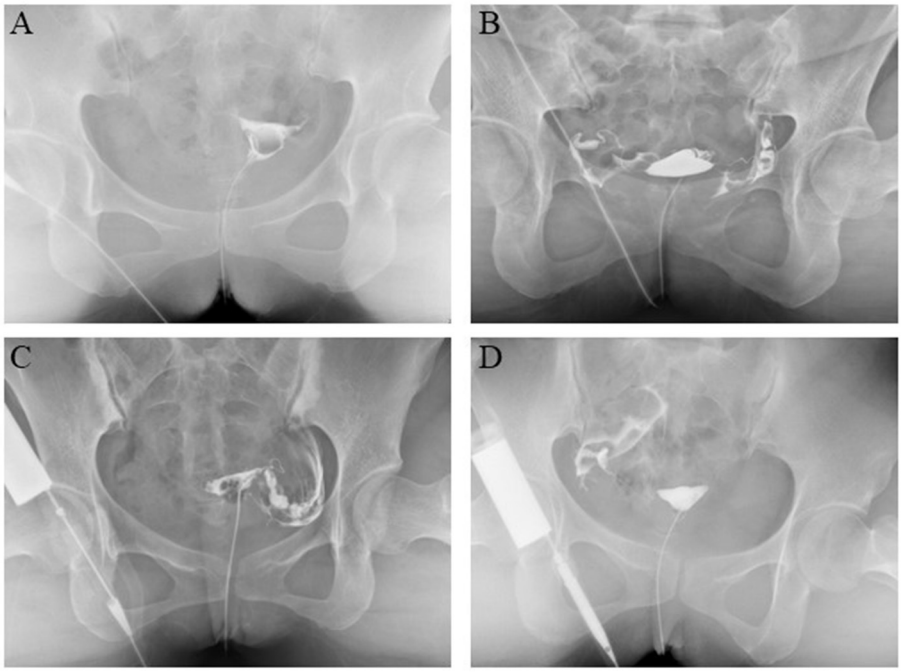

FIGURE 1 | Diagnosis of fallopian tube patency or occlusion by hysterosalpingography (HSG). **(A)** Both fallopian tube patency. **(B)** Both fallopian tube occlusion. **(C)** Left tube patency and right tube occlusion. **(D)** Right tube patency and left tube occlusion.

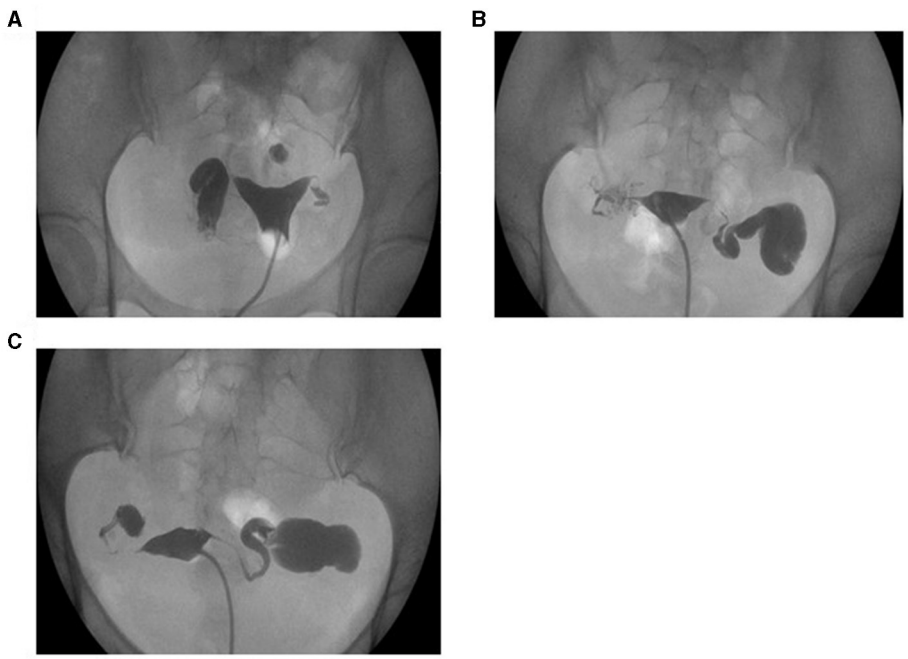

FIGURE 2 | Diagnosis of hydrosalpinx by HSG. **(A)** Right tube hydrosalpinx. **(B)** Left tube hydrosalpinx. **(C)** Both tube hydrosalpinx.

TABLE 1 | Relationship between the diagnosis of each tube by hysterosalpingography (HSG) and by laparoscopy (*n*, %).

HSG result	Normal	Occlusion	Pelvic adhesion	Total	χ^2	*p*
Right/not-so-patency	86 (41.7%)	23 (11.2%)	97 (47.1%)	206	NA	NA
Right/patency	109 (50.9%)	8 (3.7%)	97 (45.3%)	214	9.82	0.007[a]
Right/Occlusion	49 (7.9%)	330 (53.3%)	240 (38.8%)	619	19.63	<0.001[a]
Left/not-so-patency	83 (43.9%)	43 (22.8%)	63 (33.3%)	189	NA	NA
Left/patency	109 (59.2%)	15 (8.2%)	60 (32.6%)	184	17.05	<0.001[b]
Left/Occlusion	29 (4.6%)	422 (66.4%)	185 (29.1%)	636	43.84	<0.001[b]

[a]*Comparison with the not-so-patency tube of the right side.*
[b]*Comparison with the not-so-patency tube of the left side.*
NA, non-acquired.

addition, the proportion of pelvic adhesion was as high as 47.1 and 33.3%, respectively.

Compared with the right/left tube occlusion group, the diagnosis of patency/occlusion/pelvic adhesion in the corresponding right/left low patency tube by laparoscopy was significantly different with *p*-values at <0.001 and <0.001, respectively) The cases diagnosed with right/left tube non-patency by HSG tended to be expected and had minor occlusion by laparoscopy compared with the right/left tube occlusion group.

Table 2 shows the performance of HSG in the diagnosis of right tube patency or occlusion compared to laparoscopy as the gold standard. There was a high sensitivity (73.65%), specificity (83.21%), positive predictive value (50.93%), and negative predictive value (92.08%). The Kappa value was as high as 0.47, 95% CI (0.399, 0.541), $p < 0.001$. The corresponding sensitivity, specificity, positive predictive value, and negative predictive value of HSG in diagnosing left tube patency or occlusion were 78.98, 87.72, 56.19, and 95.44%, respectively. The Kappa value was 0.574, 95% CI (0.505, 0.0.643), $p < 0.001$.

Comparison of HSG and Laparoscopy in the Diagnosis of Both Fallopian Tube Patency and Occlusion

From **Table 3**, we found that when the bilateral tubes were diagnosed with patency or occlusion by HSG, the probability of bilateral tube patency or occlusion was 87.2 and 58.8%, respectively, which implied that HSG had the same diagnostic value in bilateral fallopian patency as laparoscopy. However, the diagnostic value of bilateral tubal occlusion was relatively poor. The sensitivity, specificity, positive and negative predictive values of HSG in diagnosing bilateral tube patency or occlusion were 97.94, 95.78, 87.2, and 99.38%, respectively (**Table 4**). The Kappa value was as high as 0.898, 95% CI (0.838, 0.937), $p < 0.001$.

Comparison of HSG and Laparoscopy in the Diagnosis of Hydrosalpinx

Table 5 shows that when the right/left tube was diagnosed as hydrosalpinx, the probability of tube hydrosalpinx was 79.8 and 67.9%, respectively. When the bilateral tube was diagnosed with hydrosalpinx, the chance of real hydrosalpinx was 72.4%, somewhere between the above two probabilities. The remaining was likely to be pelvic adhesion. Regardless of tube hydrosalpinx or pelvic adhesion, both factors contributed to infertility. Moreover, the kappa value of the diagnostic consistency was as high as 0.838, 95% CI (0.754, 0.922), $p < 0.001$.

DISCUSSION

Exploration of the female genital tract is one of the vital elements of infertility assessment. Laparoscopy provides a comprehensive view of the pelvic reproductive anatomy and a magnified view of pelvic organs and peritoneal surfaces (10, 11). It is generally accepted that diagnostic laparoscopy is the gold standard in diagnosing tubal pathology and other intra-abdominal causes of infertility, such as pelvic adhesion (11–13). Nevertheless, it must be taken in the inpatient department, and the patients need anesthesia. HSG is a frequently utilized diagnostic method in assessing the tubal status and detecting intrauterine anatomical defects in infertility diagnostic patients, which is convenient and safe, and less invasive. To determine the diagnostic value of HSG for infertility factors, we performed this study.

Our study found that the diagnosis of bilateral fallopian tubes as a patent by HSG was very consistent with the diagnosis by laparoscopy. It is reasonable to infer that once the bilateral tube is diagnosed with patency by HSG, the patients have a low incidence of infertility due to tubal factors. However, when the bilateral tubes were diagnosed with occlusion by HSG, there was a 27.5% chance of unilateral or bilateral fallopian tube patency. This may result from insufficiency of contrast agent influx during the angiography operation or spasms of the lower genital tracts. Therefore, the reliability of HSG is always questionable, especially for the diagnosis of tubal occlusion (7, 10, 14). In addition, there were high diagnostic values and consistency of HSG compared with laparoscopy in the diagnosis of bilateral tube patency or occlusion, which was demonstrated by the very high sensitivity, specificity, positive predictive value, negative predictive value, and high Kappa value.

The description of the degree of tubal patency by HSG has critical clinical value and can be divided into no patency, patency, and occlusion (15–17). Compared with the patency group in this study, if the tube was diagnosed with no patency by HSG, the patency or pelvic adhesion in the corresponding tube by laparoscopy was similar. Our results inferred that if

TABLE 2 | Diagnostic values of unilateral fallopian tube by HSG vs. laparoscopy.

		HSG (right fallopian tube)		Total	HSG (left fallopian tube)		Total
		Normal*	Abnormal#		Normal*	Abnormal#	
Laparoscopy	Normal*	109	49	148	109	29	138
	Abnormal#	105	570	619	85	607	636
Total	214	619	833	194	637	830	

*Normal was defined as patency with the HSG assessment or the laparoscopy assessment.
#Abnormal was defined as occlusion with the HSG assessment, or occlusion of single or both tubes, or pelvic adhesion with the laparoscopy assessment.

TABLE 3 | The relationship between the diagnosis of patency (or occlusion) of both tubes by HSG and by laparoscopy *n* (%).

	Both tubes occlusion by lap	Single tube occlusion by lap	Both tubes patency by lap	Pelvic adhesion	Total
Patency of both tubes by HSG	2 (1.20%)	0	143 (87.2%)	19 (11.6%)	164 (100%)
Occlusion of both tubes by HSG	282 (58.8%)	129 (26.9%)	3 (0.6%)	66 (13.8%)	480 (100%)

TABLE 4 | Diagnostic values of both tubes by HSG vs. laparoscopy.

		Laparoscopy		
		Normal	Abnormal	Total
HSG	Normal*	143	21	164
	Abnormal#	3	477	480
Total		146	498	644

*Normal was defined as patency with the HSG assessment or the laparoscopy assessment.
#Abnormal was defined as occlusion with the HSG assessment, or occlusion of single or both tubes, or pelvic adhesion with the laparoscopy assessment.

TABLE 5 | Consistency between laparoscopy and HSG in patients diagnosed with hydrosalpinx by HSG *n* (%).

	Hydrosalpinx by laparoscopic	Pelvic adhesion	Normal	Total
Right tube hydrosalpinx by HSG	75 (79.8%)	16 (17.0%)	3 (3.2%)	94 (100)
Left tube hydrosalpinx by HSG	72 (67.9%)	24 (22.6%)	10 (9.4%)	106 (100)
Both tubes hydrosalpinx by HSG	97 (72.4%)	36 (26.8%)	1 (0.7%)	134 (100)

Comparison of the laparoscopic diagnosis consistency between the right and left hydrosalpinx by HSG diagnosis; $\chi^2 = 4.73$, $P = 0.094$.

the tube was diagnosed as not patent, there was a more than a 40% chance for the tube to be patent. The patients could experience drug treatment or artificial insemination for their next step; in addition, the proportion of pelvic adhesions was more than one-third. Compared with the blockage group, if the tube was diagnosed with no patency by HSG, the diagnosis of patency, occlusion, or pelvic adhesion in the corresponding tube by laparoscopy was significantly different. Thus, we concluded that low patency of the fallopian tube by HSG had a specific guiding significance in infertility analysis. At the same time, we found that the diagnostic values of unilateral fallopian tubes by HSG were high through high sensitivity, specificity, and negative predictive value. However, we still kept in mind the false-positive predictive rate of single tube occlusion. In addition, the diagnostic consistency in occlusion by HSG and by laparoscopy was demonstrated by kappa values of 0.47 [95% CI (0.399, 0.541), $p < 0.001$] and 0.574 [95% CI (0.505, 0.643), $p < 0.001$], respectively, which indicated moderate strength consistency. Considering the low cost and high efficiency of HSG in diagnosing infertility, many scholars recommend HSG as an auxiliary routine outpatient examination in the assessment of infertility (7, 18).

Hydrosalpinx is the morphological change in the fallopian tube resulting from chronic inflammation stimulation (19, 20). Ultrasound and HSG help diagnose hydrosalpinx, but the exploration of hysteroscopy combined with laparoscopy was considered the gold standard for the diagnosis of hydrosalpinx, which could simultaneously inspect the situation of the pelvic cavity (21–23).

Because the peristalsis of the fallopian tube is affected by ovarian hormones (24, 25), it is difficult for ultrasound examination to differentiate hydrosalpinx and severity. With the use of a multidose contrast agent, HSG could effectively diagnose hydrosalpinx (6, 7). In our study, when the tube was diagnosed hydrosalpinx by HSG, there was an ∼70% chance accuracy; the diagnostic consistency in hydrosalpinx by HSG and by laparoscopy was very high, and the Kappa value was 0.838 [95% CI (0.754, 0.922), $p < 0.001$]. In recent years, hydrosalpinx has been one of the leading causes of secondary tubal infertility. The diagnosis and treatment of hydrosalpinx significantly impacted the natural conception and pre-treatment of *in vitro* fertilization and embryo transfer (IVF-ET). Considering that hydrosalpinx has a particularly adverse effect on the success rate of IVF-ET, we referred the patient to experience surgical treatment when HSG demonstrated the presence of hydrosalpinx.

Hysterosalpingography has a relatively low expense, but it plays an essential role in predicting the status of the fallopian tube and pelvic situation, which should be conducted as the first diagnostic procedure in assessing infertility. However, the false-positive rate of tube occlusion, correct interpretation of the report, and the course of the procedure could help us make a proper diagnosis. Many strategies could be utilized to overcome the limitations of HSG, including laparoscopy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by First Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JT and YL contributed to the design of the study. JT and MD contributed to a manuscript written and statistical analyses. MX, WP, and ML carried out data acquisition and analysis. YL critically reviewed the study. All authors were involved in and approved the final version of the article before submission.

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REVIEW
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Involving Animal Models in Uterine Transplantation

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Background: Absolute uterine factor infertility affects 0. 2% women of childbearing age around the world. Uterine transplantation (UTx) is a promising solution for many of them since the first birth from UTx was described by the Swedish team in 2014. The success of Utx in humans has become possible after a systematic and meticulous approach involving years of research on animal models. To date, more than 80 UTx procedures have been performed worldwide and 30 children were born.

Material and Method: This review summarizes the research preparation conducted in animals before beginning UTx in humans. It focuses on the advantages and limits of each animal model, their place in surgical training, and current contribution in research to improve UTx successes in humans. The different steps in the process of UTx have been analyzed, such as imaging, surgery, ischemia-reperfusion effects, rejection markers, immunosuppressive treatment, and pregnancy.

Conclusion: Animal models have played an essential role in the implementation of UTx, which is a highly complex procedure. While respecting the 3R requirements (replacement, refinement, and reduction), the surgical training using large animal models, such as notably ewes remain irreplaceable for teams wishing to initiate a UTx program. Furthermore, animal models are still mandatory in current research to improve the success rates of UTx in humans as well as to reduce the morbidity associated with this experimental infertility treatment.

Keywords: uterus, transplantation, surgery, sheep, animal, animal models

INTRODUCTION: THE SWEDISH MODEL

Absolute Uterine factor infertility affects one in 500 women of childbearing age (1). These women's options for bearing a child are adoption, surrogate motherhood, or uterine transplantation (UTx). The first livebirth after UTx in women occurred in 2014 in Sweden (2), as part of the first world UTx series. Among nine UTx performed, seven were successful and eight healthy children were born (3). This high rate of success resulted from a long and meticulous preparation.

Using an animal model is a preliminary and mandatory step to any surgical innovation as mentioned in Moore's criteria and the idea, development, exploration, assessment, long-term study (IDEAL) concept (4, 5). In 2009, the International Federation of Gynecology and Obstetrics (FIGO) recommended preliminary studies in several animal models, such as non-human primate prior to undertaking UTx (6).

The Swedish team, led by Mats Brännström, studied all aspects of UTx in numerous animal models during more than a decade before performing their first trial in humans. They worked initially on mice and rats and reported the first livebirth after UTx in syngeneic mice in 2003 (7) and after allotransplantation in 2010 (8). They subsequently worked on larger animal models, such as sows since 2004 (9) and ewes since 2005 leading to the first livebirth after auto-transplantation reported in 2010 (10). Ultimately, this team worked on baboons in 2008. Based on the experience gained using animal models, Brännström's designed a human protocol. Despite accomplishments achieved in humans, they still train regularly on sheep for practicing surgery because it is the model closest to humans.

The challenge is to stay ahead in this high-level set fertility performances while respecting the ethical aspects of animal research, represented among others by the 3R system, such as replacement, refinement, and reduction (11).

We searched articles in English in PubMed using the Keywords: "uterus," "uterine," "transplant," "transplantation," and subsequently AND mice, AND rat, AND pig, AND sheep, AND ewe, AND macaque, and AND baboon. We excluded all reviews, articles on other topics, articles not in English, articles published before 2000. We excluded some animal models because they had limited impact (rabbits, dogs, and cats).

In this review, we describe the strengths and weaknesses of the different animal models. In addition, we explain their respective contribution in different aspects of UTx notably, in terms of surgical training and research (imaging, surgery, ischemia-reperfusion, rejection, immunosuppressive treatment, and gestations). Finally, we look at some possible future research projects based on animal models.

STRENGTHS AND WEAKNESSES OF THE VARIOUS ANIMAL MODELS

Advantages and disadvantages of different animal models for UTx are described in **Table 1**.

The first successful UTx using an animal model was conducted in rodents. This species is easily available and has perfectly known physiology, immunology, reproduction, and genetics. Moreover, practicing UTx with a syngeneic model permits to avoid immune reaction (12, 13). Gestation period is short allowing an easy and repeated study. The cost of the procedure is relatively low, and studies can be made on numerous animals. The main drawback is its anatomical size. Indeed, for the surgical training, small animals are unfortunately inappropriate because of the size difference with humans. This is especially the case when vessel dissection and anastomosis are performed. It is therefore essential to practice surgery on large animals.

However, choosing the right large-animal model, however, is difficult. Studies were led on pigs (9, 14), which have similar vascular anatomy. Unfortunately, the pelvic anatomy is very different from that of humans. The uterus of the pig is composed of two long horns of 1 m each, making hysterectomy and anastomoses difficult and different from humans. In spite of these differences, some research was conducted: a heterotopic allogeneic model in mini-pigs (15) usable for basic research and an auto-transplantation model using the ovarian vein (16). Gestation is about one-third of the humans (110 days) with a large litter size. For most teams, the pig model can be used for research, but is not the right animal model for practicing transplantation surgery in preparation for the human UTx.

Ewes represent a better model than pigs because of their similarity with humans in terms of body size, pelvic anatomy, and uterine vessel size (17). It is the best animal model for surgical training in UTX (18). Uterine arterial vascularization is very similar to that of humans, but the venous drainage is quite different. There are two utero-ovarian veins in the ewe while there are two uterine veins and two ovarian veins in women (19). Furthermore, the uterus is bicornuate in ewes (**Figure 1**). The difficulty lies in the risk of ileus blockage and difficulty of recovering the rumination process. Sheep are phylogenetically relatively far from humans in terms of immunosuppression processes, making research in this field difficult. The gestation time is nearly half of that observed in human (145 days), with 1–2 fetuses of human size. This facilitates studying pregnancies and the development of lambs.

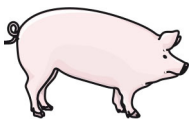
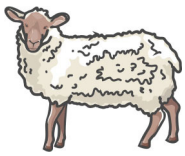
Non-human primates appear as a very good model for UTx notably, because anatomy and more specifically, vascular anatomy is very similar to that of humans. Cynomolgus and rhesus macaques (20) were used as UTx model but surgery is difficult because of the small size of macaques and their vessels. Another limitation is the reduced fertility with monthly ovulation, single and long pregnancy. Moreover, the cervix is angled, which makes cervical biopsies and embryo transfers problematic. For these reasons, trials were performed in baboons (21), that are bigger but still half the size of human beings. Their cervices are also linear. Nevertheless, even if non-human primates offer a model of choice because of their anatomical and phylogenetic similarities to humans, their use is ethically very difficult, and their cost remains high. The availability of these species for transplantation research is therefore very limited. Thus, although these were necessary before initiating the world first UTx cases, the primate model is not appropriate any more for current research or training in view of UTx.

SURGICAL TRAINING

Starting a new UTx program is a complex multidisciplinary process. UTx is more complex than other major live donor transplantation procedures.

Live donor surgery is a far more extensive procedure than a simple hysterectomy. The uterine vessels must be preserved, which enhances the difficulty and can lead to major complications. The dissection of uterine veins is complex due to

TABLE 1 | Advantages and disadvantages of different animal models for UTx.

Species		Advantages	Disadvantages
		Availability Syngeneic model Ethical acceptability Cost Fertility Short pregnancy (19–20 days)	Size Phylogenetic difference
		Size Similar uterine and pelvic vascular anatomy Availability Fertility Longer pregnancy (1/3 of human pregnancy)	Pelvic and uterine anatomy Small size of vessels Phylogenetic distance Cost Limited ethical acceptability Phylocotous
		Size Pelvic anatomy Vessel size Availability Longer pregnancy (1/2 of human pregnancy)	Venous uterine drainage Postoperative recovery Phylogenetic distance Cost Limited ethical acceptability Only one or two offspring in general
	Macaque	Pelvic anatomy Uterine anatomy	Size Small size of vessels Angled cervix Limited availability Cost Very limited ethical acceptability Limited fertility Longer pregnancy (around 180 days) Only one or two offspring in general
	Baboon	Size Pelvic anatomy Uterine anatomy Linear cervix Phylogenetically close to human	Availability Cost Ethics limitations Very limited ethical acceptability Longer pregnancy (around 180 days)

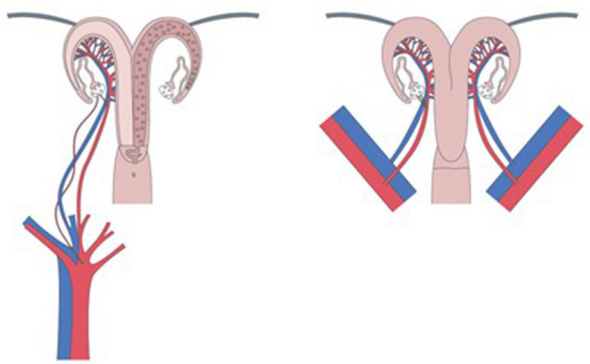


FIGURE 1 | Uterus autotransplantation in sheep.

their proximity to the ureters and the number of small venous branches. A complete ureterolysis and removal of a patch of the internal iliac vessels is also necessary. Moreover, substantial portions of the uterine ligaments, an extensive sheet of bladder peritoneum, and part of the vagina are also needed for the anastomosis with the recipient. The duration of the live donor surgery—removal of the uterus—in the hands of the high-skilled Swedish team was 10–13 h (22). The introduction of robotic surgery was shown to improve the dissection and postoperative recovery, though the surgery duration was not reduced (23).

The recipient's surgical operation is performed by laparotomy. The first step is the dissection of the vaginal vault from the bladder and rectum. The external iliac vessels are then exposed, and the uterine graft is placed in the orthotopic position with end–to-side anastomoses between uterine vessels and iliac extern vessels. The average duration of surgery for the recipient lasts close to 5 h (22). Microsurgery skills using 7.0 sutures are needed for vascular anastomoses. Transplantectomy is necessary in 20% of cases mainly due to thrombosis (3). The different steps are represented in **Figure 2**.

A meticulous preparation and optimal settings are necessary components for the responsible introduction of UTx. Moreover, prior surgical training on animal models is seminal for success.

Building a UTx-dedicated team through training on a large-animal model is mandatory before performing the UTx in humans (24). Kisu et al. noticed an 82% success rate when having gained UTx expertise in an animal model and 55.6% without expertise (25). The surgical team should involve both gynecologic surgeons with oncologic skills and transplant surgeons mastering microsurgery and robotics procedures. The ewe autotransplantation training protocol used by the authors is available in the attached video and in **Figure 3**. The different steps of the surgery, performed after laparotomy, are close to human dissection of uterine arteries and utero-ovarian veins, freeing and retrieval of the uterus, flushing vessels on Back table, and replacing it in an orthotopic position using end-to-side vascular anastomosis with external iliac vessels. One ewe is used as donor and recipient in accordance with the 3R. We found out that in ewes, achieving uterine dissection is possible after five cases and arterial and venous anastomoses are reproducible after seven and nine cases, respectively (17).

BASIC RESEARCH

The contribution of different animal models to UTx is summarized in **Table 2**.

Different questions at each step of the UTx process were provided by research in different animal models as illustrated in **Figure 4**. The selection of the candidates (compatible donors

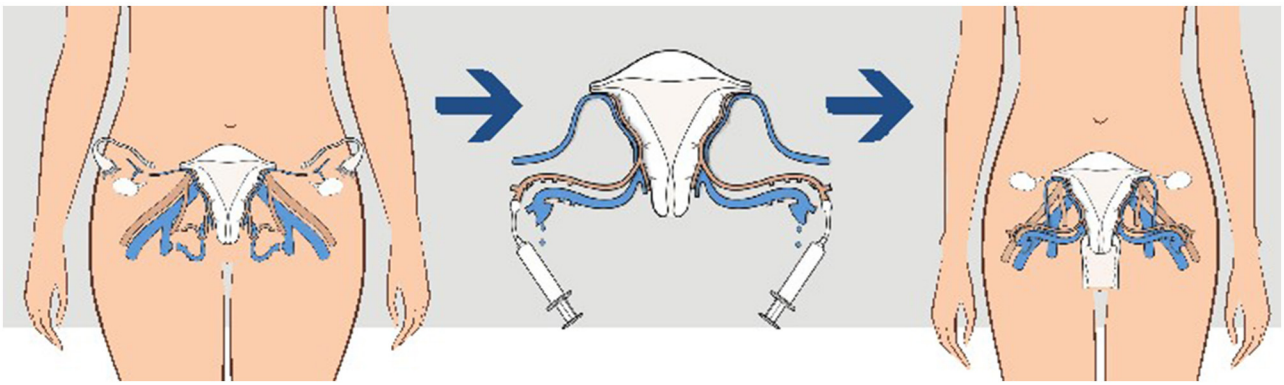


FIGURE 2 | Steps of uterine transplantation (UTx) in human.

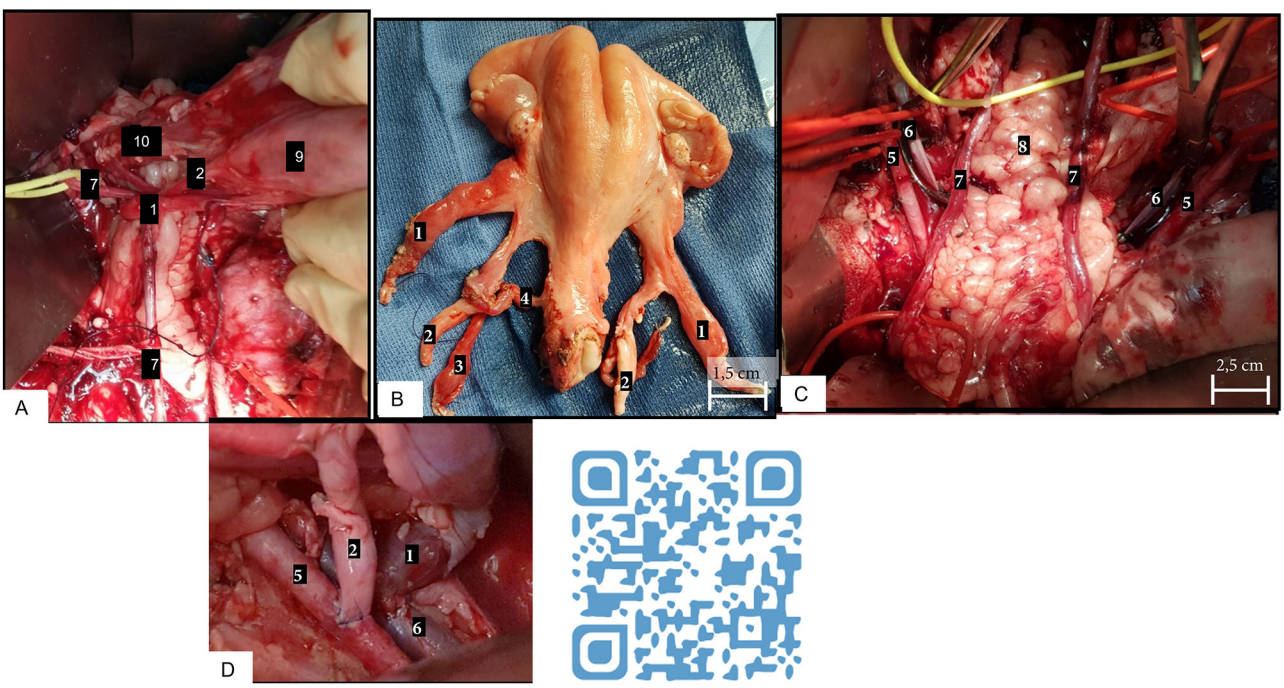


FIGURE 3 | Different steps in UTx in sheep. **(A)** Uterine dissection, lateral view. **(B)** Back table. **(C)** Pelvic view before anastomoses. **(D)** Arterial and venous latero-terminal anastomoses. (1) Utero-ovarian vein; (2) uterine artery; (3) umbilical artery; (4) cervico-uterine arterial branch; (5) external iliac artery; (6) external iliac vein; (7) ureter; (8) rectum; (9) uterine horn; and (10) cervix.

TABLE 2 | Uterine transplantation (UTx) studies in animal models.

	Selection	Transplantation techniques	Pregnancy	Ischemia studies	Rejection studies	Bioengineering
		Syngeneic heterotopic transplantation (7, 15, 26) Allogeneic heterotopic transplantation (27–29)	Achieved (7, 30) *In utero* exposure of cyclosporine A (8)	24–48 h cold ischaemic preservation (26)	Rejection patterns (27) Immunosuppression with cyclosporine A (28) Leukocyte subtypes in rejection (31)	
		Inbred heterotopic transplantation (32, 33) Allogeneic heterotopic transplantation (34, 35) Syngeneic orthotopic transplantation (13, 36, 37) Allogeneic orthotopic transplantation (38–40)	Achieved in syngeneic (13) and in allogeneic (34) Effect on tacrolimus on offspring (38)	4 h warm ischemia study (37) Remifentanil (41), Melatonin and Glycine (42) or cannabinoid agonist JWH-133 (43) decrease ischemia-reperfusion injury Using Histidine-Tryptophan-Ketoglutarate with acetyl L-carnitine (44) or Custodiol-N (45) for uterus preservation	Immunosuppression (34) Effects of cyclosporine A (39) Effects of tacrolimus (40)	Uterus decellularization (46–48) and recellularizing (49, 50) Bioengineered patches in decellularized uterus permits pregnancy (51) Immune response in transplanted decellularized uterus (36)
		Auto-transplantation (9) Heterotopic (15) and orthotopic (52, 53) allogeneic transplantation		Early reperfusion events (9)	Immunosuppression with tacrolimus and cyclosporine (15)	
	MRI study (54) Multispectral imaging laparoscopy (55) Indocyanine green angiography (56) Angiography (57)	One horn auto-transplantation (58) Orthotopic auto-transplantation (10, 17–19, 55, 59–63) and allotransplantation (64, 83) Laparoscopic auto-transplantation (57)	Achieved in auto-transplantation (64) and allo- transplantation (10)	Early reperfusion events (19, 58, 65) Long cold ischemia evaluation (18, 63) Normothermic *ex vivo* reperfusion model (66)	Immunosuppression with cyclosporine (64)	Uterus decellularization (67) Recellularization in bioactive uterus scaffolds (68)
Baboon						
		Autotransplantation (69) Autotransplantation with end-to-side or end-to-end anastomoses (21) Autotransplantation with utero-ovarian anastomoses only (30) Autotransplantation using uterine and ovarian pedicles (70) Allogeneic orthotopic transplantation (68, 71)	Achieved in autotransplantation (30)	Long-term reperfusion (69)	Immunosuppression protocol (71) Long-term graft survival (72)	
Macaque						
	Indocyanine green angiography (73, 74)	Autotransplantation (73, 75) Orthotopic allotransplantation (20, 76)	Achieved in autotransplantation (20) and allotransplantation (77)	Evaluation of warm ischemia (78)	Immunosuppression protocol (79, 80)	

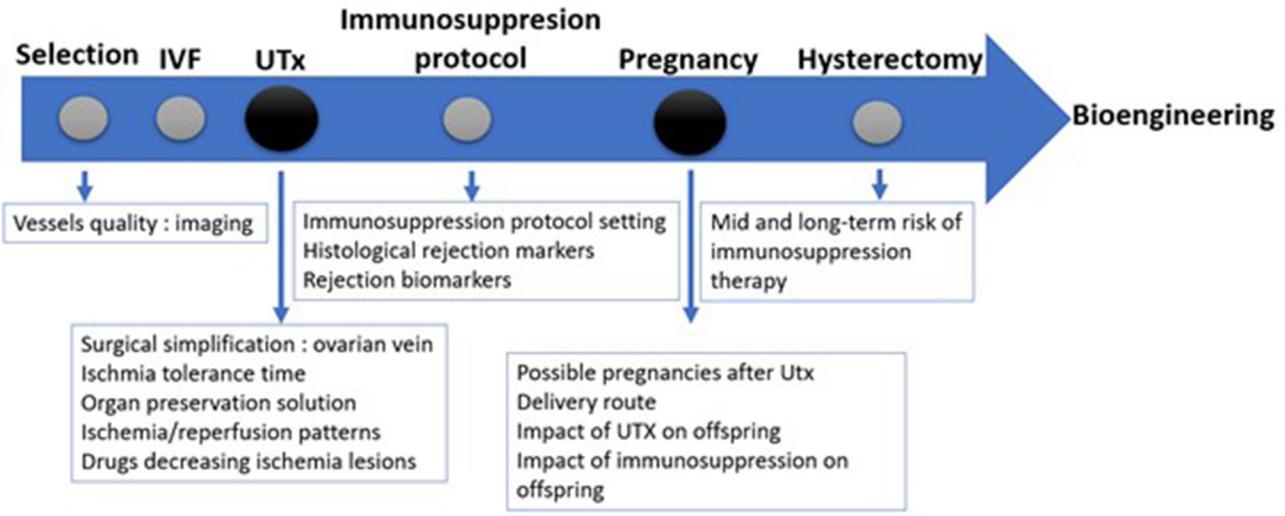


FIGURE 4 | Different steps of UTx and their research domains. IVF: *In vitro* Fertilization.

and recipients) is the first step. The use of appropriate imaging is mandatory to assess the vessel quality. Before surgery, *in vitro* fertilization (IVF) is performed to obtain enough embryos as fallopian tubes are removed during surgery due to devascularization. Afterward, UTx is realized. Immunosuppression is introduced right away and after assuring that there is no rejection, the embryo can be transferred. Delivery is achieved *via* caesarean section and the uterus is removed after one or two pregnancies.

Selection: Imaging

Selection of the donor is essential and requires imaging to assess the quality of the graft and its vascularization preoperatively as postoperatively. There are no studies using animal research that looked at CT angiography (CTA), digital subtraction angiography (DSA), and magnetic resonance angiography (MRA) to preoperatively evaluate the "quality" of uterine arteries. Imaging was, however, used in animals to ascertain the quality of the vascularization of the graft after surgery.

A Doppler examination is often used pre- or postoperatively to visualize the blood flow in the transplanted uterus (17). An implantable Doppler Cook-Swartz has been used in ewes (56) to monitor the blood flow postoperatively. This invasive technique used a small ultrasound sensor fixed near the anastomosis to ascertain its permeability.

In addition, MRI was used in ewes, first to define the surgery's feasibility (54) and second (81) to postoperatively analyze the graft. Coupled with ultrasonography, the measurement of uterine size as well as assessment of the presence or absence of graft edema or of an eventual thrombosis of the anastomoses were enabled.

Indocyanine green (ICG) is injected intravenously and identified with a special camera obtaining images of very small vessels intraoperatively. The value of the technique has been proved in the ewe (56) and macaque (73). It can reveal the vascularization of the donor and so show possible anatomical variations. ICG angiography proved that a unique uterine artery on one side in macaque vascularizes both sides of the uterus, cervix, and oviducts (74), while one ovarian artery do not vascularize the cervix and contralateral oviduct. In the ewe (56), ICG angiography enabled evaluation of the permeability of anastomoses and could be repeated when a stenosis is noticed.

Additionally, the multispectral imaging laparoscopy has been used in an autotransplantation ewe model to visualize the organ oxygen saturation during the procedure (82).

In human research, arteriography is the best imaging approach to assess vessels before performing UTx (83). Further studies in animals should be conducted to test other less invasive imaging techniques than arteriography before UTx for evaluating uterine arteries and try to find an appropriate way for imaging veins, as nothing is available to date.

Surgery

Several surgical techniques were developed to simplify the UTx procedure. The aim of these simplifications was to either reduce the surgical time or test new surgical techniques before implementing them in humans.

In rodents (15, 32), due to the small vessel size, aorta and vena cava or common iliac vessels are collected to perform UTx forcing the sacrifice of the donor. The vessels are anastomosed to the aorta and caudal vena cava of the recipient. This surgical technique enabled much needed basic research. In larger animals, end-to-end anastomoses are performed in a few cases in pigs (9) and in sheep (84). A deceased donor model in sheep was also developed (81), using end–to-side anastomosis of aorta and cava inferior patch to the external iliac vessels.

To use a deceased donor, a technique of perfusion *via* the femoral and/or external iliac artery in the macaques has shown good perfusion of the uterus. This suggests that it is possible to use this perfusion technique in UTx for brain-dead donor

TABLE 3 | Classification of acute uterine rejection in endocervical biopsy samples in baboon (71).

Grade	Rejection	Biopsy findings
0	No	Normal morphology
1	Mild	Mild diffuse mixed inflammatory cell infiltrate (mainly lymphocytes). Occasional epithelial apoptotic bodies, focal distribution. Surface epithelium intact. No necrosis
2	Moderate	Moderate, diffuse mixed inflammatory cell infiltrate (mainly lymphocytes). Increased amount of epithelial apoptotic bodies: Reduced thickness surface epithelium, possible focal erosion. No necrosis
3	Severe	Significant, diffuse and aggregate, mixed inflammatory cell infiltrate (mainly lymphocytes; neutrophils and eosinophils may be present). Frequent apoptotic bodies. Epithelial erosions, focal to total. Focal necrosis
4	Total necrosis	Necrotic tissue only

instead of classical aorta canulation incompatible with the uterine preservation (85), as successfully demonstrated in humans (86). In sheep (17, 19, 55), macaque (74, 85), and baboon (69), mostly end-to-side anastomoses between uterine vessels and external iliac vessels were performed, which is the surgical technique used in humans. To simplify UTx, some teams used only one-sided anastomoses. Although the graft viability was obtained with this technique, organ perfusion was significantly reduced. A bilateral revascularization of the graft was therefore necessary to allow gestation on a transplanted uterus (20, 87).

In ewes, ovarian veins (17, 19, 55) were used and short-term drainage of the uterus was feasible through a single venous anastomosis (65). A model in macaque showed that using only the ovarian vein compared with the deep uterine vein was less invasive (88). The outcomes in both groups in terms of vascular injuries and uterine function were similar.

This model was translated in humans and showed favorable issues with functional uterus and live birth (89). A uterine autotransplantation in baboons showed good results when using ovarian veins (70). Furthermore, a description of live births in baboons with an angiosome using microsurgically anastomosed utero-ovarian vessels and lacking uterine arteries and veins is a promising research for reducing morbidity of the surgery for donors in humans (30).

Ischemia-Reperfusion

Ischemic injuries occur when the grafts stopped being longer perfused. There are two different types of ischemia: warm and cold. Both are responsible for uterine injuries on all levels: molecular, cellular, and tissue. The ischemia-reperfusion syndrome can lead to acute graft rejection (90). In ovine model, several markers for ischemia-reperfusion were searched using the histological, immunohistochemistry, and molecular biology approaches (17, 19, 65). The increase in some ischemia markers was due to oxidative stress and inflammation induced by ischemia-reperfusion (65).

Warm ischemia can rapidly induce uterine damages. Histopathological changes due to warmth ischemia were studied in cynomolgus monkeys (78). Warm ischemia can cause permanent damage if it lasts more than 4 h. Within 3 h, there were no histological or functional injuries, and these results that are transposable to the humans. In rats, the injection of Melatonin and Glycine (42), Remifentanil (41), or cannabinoid agonist JWH-133 (43) reduced warm ischemia-reperfusion injuries.

The uterus can tolerate up to 24 h of cold ischemia in mice (26). Grafts were preserved 24 h in a heparinized isotonic saline solution and transplanted successfully. The results showed a good uterine resilience after a long time of cold ischemia. A reperfusion model in ovine (66) was developed to evaluate the tolerance of the uterus for increased cold ischemia times. Uteri were placed for 4–48 h in cold ischemia and reperfused for 48 h in normothermic conditions with a sterile solution which composition was close to blood whereas the temperature and oxygen level can be modified. This showed favorable outcomes with normothermic *ex vivo* reperfusion and allowed to study ischemia-reperfusion markers *ex vivo*.

Several preservation solutions were used in animal models to improve the quality of the grafts. Custodiol-N (45) was better than Custodiol for uterine preservation in rats. Adding acetyl L-carnitine to a Histidine-Tryptophan-Ketoglutarate solution prevented the formation of free radicals (44). Perfadex® (10) containing colloid and dextran 40 was better than saline solution for UTx in sheep. By preventing the interaction of activated neutrophils with the vascular endothelium, it protects microvascularization from inflammatory lesions and prevents edema formation during the graft preservation.

Uterine function is dependent upon ischemia-reperfusion injuries. Further basic research on intra- or postoperative therapies leading to a reduction of these lesions will improve the chances of success of this difficult surgery. The challenge for reducing ischemia is mandatory and, there are two ways to do so: improve surgery and find treatments.

Graft Rejection

Similar to other kinds of transplantation, the uterus can present acute or chronic rejection. Diagnosis of rejection in transplanted organs is made when its functionality decreases leading to several symptoms and blood markers. Uterus rejection is difficult to diagnose because there are no symptoms or blood test expressing uterine functionality. The classification for acute forms uterine rejection in baboons is illustrated in **Table 3** (71), which led to establish the classification that is now used in human UTx.

Non-invasive rejection markers need to be found to facilitate the follow-up of transplanted patients.

Immunosuppressive Protocol

Immunosuppressive drugs are mandatory to avoid organ rejection in every allotransplantation. After achieving syngeneic UTx, allotransplantation comes with immunological challenges.

The first allogeneic transplantations (27) were achieved in mice and showed the need for immunosuppressive therapy. Without treatment, the inflammation was visible 2 days after transplantation. After 10–15 days, inflammation was maximal, leading to necrotic changes in the graft 28 days after transplantation. Thereafter, cyclosporine A was used in the same model as immunosuppressive therapy. This drug (28) delayed but

did not stop uterine graft rejection in the mid and long course. Moreover, high doses of cyclosporine A (13) reduced the embryo implantation rates and increased the fetal mortality rates. One live birth in an ovine model was obtained with cyclosporine A treatment (91).

Fujimycin was used in a rat model and several full-term gestations were obtained with this treatment. There was, however, a significant failure rate requiring further research (34). A triple immunosuppressive therapy (fujimycin, cyclosporine, and methylprednisolone) enabled a 50% long-term survival rate in a mini-pig model (15). The phylogenetic distance of these models from the human species, however, did not allow the extrapolation of the obtained results.

The first immunosuppressive protocol used in a non-human primate allogeneic UTx was published in 2013 (71). Fujimycin, mycophenolate, and corticoids were combined after proving that a monotherapy of fujimycin was insufficient. In macaques, a protocol using antithymocyte globulin, cyclosporine, tacrolimus, mycophenolate mofetil, and methylprednisolone was used but found to be not enough effective (79). When rituximab was added to this protocol, it enabled a good immunological control (77).

These studies need to be considered with precaution because the human immune response can differ from non-human primates. Research in organ transplantation demonstrated the efficiency of some immunosuppressive protocols in humans whereas they were only partially effective in monkeys (92). More research on immunosuppressive protocols is thus needed to bring the right balance between immunosuppression and complications.

Pregnancy

Uterine transplantation makes sense if the transplanted organ can support the development of the embryo and allow its development to term. From the transplanted uterus, this implies the resumption of endometrial receptivity and a vascularization compatible with placental development and adequate vascularization to allow placentation and proper exchanges with the fetus.

In mice (7), gestation was obtained after embryo transfer in a syngenetic heterotopic model. The offspring of transplanted dams had the same development, growth or fertility as compared with control. A syngenetic orthotopic UTx model in rats (13) has also enabled gestation. There was more miscarriage in the transplanted animals: out of eight gestations, only one led to a successful vaginal birth, with numerous dystocia. This suggested the need of caesarean section for delivery after UTx. In rats, two male rats were born after UTx and their development was normal. Gestation was achieved in an orthotopic allotransplantation in rats (34, 38) with a higher miscarriage rate in transplanted animals. There were discordant data about the number of offspring at birth. In the first study (34), the number of raccoons was lower in the transplanted group, but in the second (38), the opposite was seen. Delivery occurred by caesarean section. The male raccoons from the transplanted uteri were larger than controls but there was no difference in female raccoons.

The first gestation in a UTx sheep model (13) occurred after autotransplantation. The gestation rate was similar in the transplanted group compared with controls. Three gestations resulted in the birth of two lambs, born by C-section, in good health. Uterine torsion was diagnosed in the third gestation, demonstrating the necessity of a good uterine ligament fixation.

There were gestations using an ovine model of allotransplantation in 2011 (91). Twelve ewes underwent transplantation and five could be selected for gestation. Frozen embryos were transferred in two ewes, one of which failed to be pregnant and the other had an extrauterine pregnancy. Fresh embryos were transferred to three other ewes, of which one did not get pregnant, one had a miscarriage, and one went to term. The lamb was born by the caesarean section 10 days before term. The analysis of its vital parameters showed no difference with those published in other studies on preterm lambs.

Gestation was first achieved in 2012 in an auto-transplantation model (20) in the macaque. An emergency caesarean section was performed because of placental abruption. The baby macaque showed signs of fetal distress and was not reanimated for ethical reasons. Normal fetal development was observed at post-mortem examination. Pregnancy was achieved lately in the macaque uterus allotransplantation (77). Gestation was attempted for three macaques in allotransplantation but only one could give birth after 3 miscarriages. A caesarean section was performed, and the offspring showed good development.

Normal pregnancies are possible in all animal models, but transplanted uterus bring more miscarriages. There are cases of fetal distress, uterine torsion, extrauterine pregnancy, and the need to practice a caesarian section to give birth.

More research is needed nowadays to identify the factors in UTx that are associated with uncomplicated pregnancies and livebirths. Among these factors, the impact of immunosuppressive treatments and UTx itself on long-term development in offspring is still poorly investigated and further studies are needed. Small models, such as rodents can be the prefect model because gestation time is very short and so long-term follow-up is possible.

DISCUSSION AND PERSPECTIVES

Lately bioengineering has been given new perspectives for uterine replacement (93). Indeed, it should be possible to create an artificial organ using a three-dimensional scaffold that would be cellularized with stem cells. This would make immunosuppressive therapy unnecessary.

Experiments on uterus decellularization were performed in rats (36). A patch of collagen and bone marrow-derived mesenchymal stem cells was inserted in a severely injured uterus. This patch has allowed an increased ability of endometrium, uterine muscle, and microvasculature regeneration (94). A matrix has been partially decellularized and has been transplanted (51). This transplanted uterus gave birth, but placentation occurred only on the cellularized patches.

Lately (95), a pregnancy was carried out using a rabbit model with an autologous cell-seeded engineered uterus. These uteri allowed fetal development and livebirth.

CONCLUSION

The use of animal models has played an essential role in the implementation of UTx in humans. Surgical training using large animal models is mandatory for any team wishing to initiate an UTx project. Research using animal models aiming at simplifying this complex new procedure is still necessary using animals even if 3R must be respected as much as possible. Hopefully, bioengineering will come up with an artificial uterus model, which will 1 day bring us the perfect mean for freeing us from animal models.

AUTHOR CONTRIBUTIONS

AF-I, MC, and JD wrote the manuscript. AF-I and JD collected the data. NC, SC, RC, FV, VG, LG, CR, HT, OS, DdZ, PC-P, and J-MA read and edited the manuscript. All authors approved the submitted version.

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REVIEW
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Artificial Ovary for Young Female Breast Cancer Patients

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In recent decades, there has been increasing attention toward the quality of life of breast cancer (BC) survivors. Meeting the growing expectations of fertility preservation and the generation of biological offspring remains a great challenge for these patients. Conventional strategies for fertility preservation such as oocyte and embryo cryopreservation are not suitable for prepubertal cancer patients or in patients who need immediate cancer therapy. Ovarian tissue cryopreservation (OTC) before anticancer therapy and autotransplantation is an alternative option for these specific indications but has a risk of retransplantation malignant cells. An emerging strategy to resolve these issues is by constructing an artificial ovary combined with stem cells, which can support follicle proliferation and ensure sex hormone secretion. This promising technique can meet both demands of improving the quality of life and meanwhile fulfilling their expectation of biological offspring without the risk of cancer recurrence.

Keywords: breast cancer, artificial ovary, fertility preservation, cancer recurrence, follicle, stem cell

INTRODUCTION

Breast cancer (BC) is the most widespread cancer in female worldwide (1, 2). The incidence of this cancer has remarkably increased since the 1970's, with the greatest boost in patients of reproductive age (3). Owing to the diagnostic and therapeutic advances, the mortality rate of women with BC is decreasing yearly (4). The long-term side effect of BC treatment is impaired or even loss of reproductive function. Premature ovarian failure (POF) may lead to insomnia, vasomotor symptoms, and osteoporosis and significantly disturbs mental function that determines the quality of life (5). Therefore, the greatest concern of these young survivors is to preserve and maintain their fertility (6).

RISKS ON OVARIAN FUNCTION DURING THE TREATMENT OF BC

First, surgical treatment may directly cause the loss of ovarian function. For BC with BRCA mutation carriers, bilateral salpingo-oophorectomy is recommended by the American College of Obstetricians and Gynecologists (ACOG) for reducing the risk of ovarian cancer (hereditary breast-ovarian cancer syndrome; HBOC) (7). Because BC with a positive BRCA mutation has a greater impact on ovarian reserve after chemotherapy treatment (8). Second, chemotherapy has genotoxic side effects. It has immediate and long-term side effects on ovarian function. A woman has finite primordial follicles (about one million) derived from the proliferation of primordial germ cells (PGCs) in their ovaries at birth, that is called a resting pool. But 85% of follicles in the resting pool are atresia before birth. Primordial follicles are recruited and activated to grow from the resting pool, most of them gradually to be atresia, and eventually, only one will ovulate during every menstrual cycle. When primordial follicles are <1,500 in the resting pool, this woman may quickly undergo menopause and lose ovarian function (9). The first effect of chemotherapy on the ovarian is immediate. It is cytotoxicity to dividing cells, which may directly kill growing follicles and induce POF. Chemotherapy may also induce inflammation and destruction of vascular and stroma, which is harmful to the growth of the follicle. However, as long as there are enough primordial follicles in the resting pool, this phenomenon can be reversible after the cessation of chemotherapy (10). Another relative side effect of chemotherapy is the long-term effect on the resting pool. The acute decrease in growing follicles, which leads to the reduction of sex steroid hormones and inhibin, may activate primordial follicles in the resting pool, enhance the rate of recruitment, accelerate the depletion of the reserve, and finally lead to POF (6).

The side effect caused by chemotherapy is dependence on the drug category used, the total dose given, and the duration of treatment. Alkylating agent is the strongest gonadotoxic drug that is widely used in BC chemotherapy. Cyclophosphamide is an alkylating agent, due to its similar DNA interstrand crosslinking agents, which can block the division of cells. Cyclophosphamide also may induce the expression of H2AX, which can break the double-strand DNA of follicles (11). Doxorubicin (adriamycin) can cross the physiological barrier of the follicle, directly acts on the DNA of oocytes, and induces cell apoptosis. Moreover, follicles in the germinal vesicle (GV) stage were more vulnerable to this toxic effect (12). Antimetabolite cytotoxic drugs often used for BC therapy, for example, fluorouracil and epirubicin, which are specific to the S-phase of the cell cycle (DNA synthesis), and have a high risk of ovarian toxicity (6).

Thirdly, radiotherapy has detrimental effects on ovarian function. Follicle is strongly sensitive to ionizing radiation. It can directly or indirectly impair ovarian function. When radiation targeted the pelvis, abdomen, or total body, it will directly impair fertility. When ovary is put away from the radiation range, some escaping radiation will be scattered and will indirectly impair fertility (10). The frequency of POF after radiotherapy is related to the used dose of radiation. Whole irradiation doses at 3–5 Gy, 60% of the follicles are destroyed; with irradiation at doses of 5 Gy, 100% of the follicles are destroyed (10). When at doses of 20 Gy, 71% of women during childhood failed to enter puberty (13).

Finally, risk of POF caused by chemotherapy is dependence on the female's age at breast cancer treatment. More than 80% of childhood cancer survivors have long-term survival into adulthood. These survivors have a 1.48-fold higher risk of POF than their siblings (14). Anti-Müllerian hormone (AMH) was detected falling rapidly in both prepubertal and pubertal girls undergoing cancer therapy (15). Another risk of POF is age-related resting pool decline on the number of primordial follicles. Because female cancer survivors are often advised to postpone pregnancy due to the risk of recurrence. For example, BC survivors with hormone receptor-positive are advised to delay pregnancy for up to 10 years after chemotherapy (6).

STRATEGIES FOR FERTILITY PRESERVATION

Medical Gonadoprotection

Medical gonadoprotection through ovarian suppression using GnRHa (gonadotropin-releasing hormone agonists) can inhibit the maturation of oocyte. Its molecular structure is similar to native GnRH but has a higher affinity to receptors. In the beginning, it can flare up the ovarian hormone secretion (LH, FSH). After 7 days, the reduction of functional GnRH receptor may decrease the release of LH and FSH, which leads to the decrease of primordial follicles' recruitment and development. So, GnRHa administer should start 7 days before chemotherapy and continues until the end of therapy (16). The decrease of ovarian hormone secretion can downregulate blood supply to the *utero*-ovarian, thereby reducing the drug entering the ovaries. The use of GnRH analogs to protect ovarian function during chemotherapy treatment is controversial (17, 18). It can interfere with anticancer therapy (19), and it also may induce reversible menopausal symptoms (20). An analysis by Lambertini et al. showed a higher pregnancy rate in women undergoing chemotherapy combined with GnRHa. But this result is still not ideal, the pregnancy rate in the chemotherapy-GnRHa group is only 9.2%, whereas in the chemotherapy-alone group is 5.5% (21). Hence, for patients with BC undergoing fertility preservation, GnRHa can only be used as an additional treatment to oocyte–embryo cryopreservation, but it cannot replace it.

Abbreviations: POF, premature ovarian failure; BC, breast cancer; ER, Estrogen receptor; ACOG, American College of Obstetricians and Gynecologists; HBOC, hereditary breast-ovarian cancer syndrome; GV, germinal vesicle; GnRHa, gonadotropin-releasing hormone agonists; OTC, ovarian tissue cryopreservation; COH, controlled ovarian hyperstimulation; 3D, three dimensional; ICSI, intracytoplasmic sperm injection; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; PGCs, primordial germ cells; PGCLCs, primordial germ cell-like cells; OSCs, oogonial stem cells; VSEL, very small embryonic-like; SSCs, skin-derived stem cells; bFGF, basic fibroblast growth factor; S1P, sphingosine−1-phosphate; SCID, severe combined immunodeficient.

Oocyte–Embryo Cryopreservation in Patients With BC

Although young women with BC face challenges in fertility, there are still many data showing that patients with a history of BC successfully conceive and do not relapse, even in patients with BC with estrogen receptor (ER)-positive (22) or germline BRCA mutations (23). Fertility restoration by oocytes and embryos cryopreservation should be highlighted for young BC women before anticancer therapy. Many studies have shown that the storage duration of cryopreservation had no negative effects on clinical outcomes (24, 25). Additionally, the pregnancy rate in frozen-thawed embryo transfer is even higher than fresh embryo transfer (26). It is established that fertility preservation and reproducible method can be safely and efficiently without being interfered with by anticancer treatments (27, 28). But it has some limitations. For embryo cryopreservation, it requires sperm to fertilize, which is difficult and unacceptable for single BC women. Oocyte and embryo cryopreservation need ovarian stimulation to retrieve mature oocytes, which may be considered contraindicated for patients with BC due to its high levels of estradiol generated by stimulation. Ovarian stimulation is also not feasible for patients with BC in childhood or prepubertal girls. In addition, the ovarian stimulation cycle usually takes 7–14 days, and there is a risk of ovarian hyperstimulation. If ovarian hyperstimulation occurs, it will take another 7–14 days to recover. These may delay the timing of anticancer treatment, which is not suitable for patients with who need immediate anticancer treatment (29).

Ovarian Tissue Cryopreservation

Ovarian tissue cryopreservation (OTC) before anticancer therapy and autotransplantation after healed is an emerging and successful method for young BC females that has produced more than 180 babies (16). OTC is a surgical method that can be carried out at any stage of BC, it not only can preserve fertility, but also restore endocrine function, produce a natural level of hormones, and have been considered as an established strategy for young patients with BC in many countries (30). OTC does not need ovarian stimulation nor require sperm and can be performed in aged 0–40 years, especially for children without delay the timing for anticancer therapy. Gellert et al. review data about 328 women who underwent autologous retransplantation of ovarian tissue, nearly 95% restored hormonal function, 72% recovered fertility function, and 40% were pregnant (31). Pacheco et al. also recorded a 65% of endocrine renovation and produced a 37% of pregnancy rate in patients with OTC and autotransplantation (32). The pregnancy and birth by OTC are increasing steadily and are exceeded 200 of live birth (33).

The risk of reimplanting residual neoplastic cells in ovarian tissue is a major safety issue (34). Ovary contaminated by BC is not uncommon, and nearly 13–47% of BCs have ovarian metastases (35). These cases were asymptomatic and often diagnosed accidentally based on autopsy or ovarian surgery, which suggests that the incidence of ovarian metastasis was underestimated (36). Both invasive lobular carcinoma and invasive ductal carcinoma in BC were reported about BC cells metastasizing to the ovaries (35). Besides, ER-positive BC and BC with axillary lymph node metastasis are positively correlated with ovarian metastasis (36). Furthermore, BC at stages III–IV and inflammatory BC are more likely to have ovarian metastasis (37). Hence, the OTC strategy for fertility preservation in young BC females should be aware and handled with caution due to the higher risks of ovarian metastasis and cancer recurrence. In these patients, the emerging technology of artificial ovary which can be an ideal alternative strategy to preserve and restore fertility should be emphasized.

Artificial Ovary

Considering the risk of reimplanting the metastasis BC cell by autotransplant OTC, artificial ovary as a promising fertility-restoring alternative approach has been investigated by many research groups from worldwide (38, 39). Although this strategy remains challenging for clinical use, promising results have been reported in animal models. Laronda et al. isolated follicles from cryopreserved human ovarian tissues to form an artificial ovary and transplanted them into ovariectomized adult mice. A number of 6 out of 7 ovariectomized mice with artificial ovary implanted had recovered hormone cycle in 4 weeks (38). Kniazeva et al. extracted follicles from young female mice and encapsulated them into an artificial ovary, mice for subsequent transplantation and mated. Nearly 33% of female mice deliver offspring (39). The main target function of the artificial ovary is to prevent reimplantation of malignant BC cells and mimic the function of the ovary. It can offer BC women opportunities to have their genetic offspring and recover endocrine function without cyclic hormone replacement therapy.

Management for Creating a Safe Artificial Ovary

Breast cancer cells spread through lymphatic and blood vessels to invasive the ovaries and colonize (37). Follicles in ovary are surrounded by a basement membrane as a protective barrier to avoid direct contact with blood vessels, capillaries, and white blood cells which can protect follicles from being contaminated by malignant BC cells (34). Follicles also is a functional unit in ovary secreting hormones and regulating the menstrual cycle. Hence, preserving follicles is a fundamental part of safely preserving reproductive function. Fortunately, primordial follicles in resting pool population in the outer cortical region of ovary and these stages of follicles are most stable for cryopreservation due to the absence of spindle, zona pellucida, and the smallest of follicular size (40). Theoretically, a small biopsy of ovarian cortex is enough for cryopreservation, because there are numerous follicles in the resting pool in the cortex. But to increase the success rate of fertility preservation, 1/2–2/3 of the cortex from one ovary should be cryopreserved in BC cases (41). Therefore, ovarian cortex cryopreservation is stable, and its isolation for retrieval follicles is a safe and well-preserved fertility function without metastasis by BC malignant cells.

To retrieve the most quality and quantity of follicles, several follicle isolation methods have been proposed and tested due to the fibrous structure of ovarian cortex. The mechanical isolation method was first used for follicle isolation and is the best method to preserve the morphology of follicles. It can generate follicles with intact basement membrane and less granulosa cell loss. But mechanical isolation is a laborious process that takes a long time, only a small part of the follicles can be isolated, and most of them remain in the tissues (42). Enzymatic digestion is an alternative approach including collagenase, Liberase, and TDE enzyme can isolate the greatest quantity of follicles, but most of them were granulosa cell lost or membrane damaged because the enzyme can digest the extracellular matrix and degrade the basement membrane (43, 44). The most effective method is the combination of mechanical isolation and enzymatic digestion yielding high quality and quantity of follicles (45, 46). Chiti et al. use a modified protocol by filtering the digestion solution every 30 min. After filtering, the isolated follicles were picked up and the remaining fragments were redigested until completely digested. This modified protocol can fully digest all types of ovarian tissue with a good preserve of isolated follicles from prolonged exposure to enzyme solution which may toxic and damage for follicles (46).

Ensuring the safety of the follicular isolation procedure without metastasis by malignant cells is a crucial step for the artificial ovary because both follicles and BC cells are involved in the digested solution. During the follicular retrieval process, follicles may also contaminate by malignant cells and replant into the artificial ovary. Soares et al. transplanted 100 leukemic cells inside an artificial ovary and grafted it into mice, none showed any sign of leukemia after 20 weeks, with reassurance by IHC and PCR method which showed all negative in the recovered ovary. It appears that grafting 100 leukemic cells is insufficient to induce leukemia (47). Meanwhile, further verified through 3 washes of follicles can effectively eliminate malignant cells without affecting the viability of follicles (48), and repeated experiments by multicolor flow cytometry (MFC) also confirmed this result (49).

Management for Creating a Functional Artificial Ovary

Folliculogenesis is a complex regulated by interaction among follicles, ovarian cells, and environmental (50). Mimicking the natural environment of the follicle to support follicle survival and development is vital for creating a functional artificial ovary. Isolated primordial follicles that are fragile need a scaffold to support three-dimensional structure (51). Interestingly, isolated follicles cultured in alginate scaffold together with theca and stromal cells had higher survival and development rates, which indicate that extracellular scaffold together with other cells as their native tissue microenvironment benefits follicle growth. Growing follicles with multilaminar structures can grow up in such tissue engineering scaffolds (52). Hence, for constructing an artificial ovary, we need a suitable scaffold that can maintain follicular three-dimensional structure and with other cells or factors which could allow follicle-cell-matrix dynamic signal communications to lead to an ovary-like environment (**Figure 1**).

Design a Suitable Scaffold for Artificial Ovary

The ultimate goal of artificial ovaries is to be retransplanted into the human body, so its ingredients must be biosafety and tolerable by the human body. The diameter of follicles' folliculogenesis from the primordial stage is 19–30 μm to the mature stage 100–110 μm, so this scaffold should be degradable for follicle growth and migration. Additionally, it also should be high-temperature resistance due to the human body temperature (34). In addition, follicles need signal communication with cells and their environment, and this 3D matrix should be high penetrated to allow the diffusion of nutrients and signal pass in and out the scaffold. Isolated follicles were fragile but it is stable while embedded in a 3D scaffold and is convenient and safe to manipulate and handle without disrupting the follicular structure (53). Overall, this 3D scaffold should be (i) biosafety and tolerable by the human body, (ii) resistant to the human body temperature, (iii) liable for cell adhesion, proliferation, and differentiation, and (iv) allow the dissemination of nutrients, growth factors, and oxygen. Tissue engineering using biomaterial supporting artificial ovary varies from natural (collagen, plasma clot, alginate, fibrin, decellularized tissues, etc.) to synthetic polymer (polyethylene glycol, 3D printing ovary, etc.) with promising and encouraging outcomes conducted in animal research models. Natural polymers are not rigid enough to support the mechanical structure, but it is superior for cell adhesion, migration, and signal communication. Synthetic polymers are superior for supporting mechanical properties when grafted in the human body, but they lack molecules for cell adhesion which is not conducive for nutrient exchange and signal crosstalk (54).

Collagen and plasma clot was the first natural scaffolds used to embedded isolated primordial follicles. Telfe et al. isolated follicles from mice and cultured them in a collagen matrix for 5 days and then grafted them to ovariectomized mice. Follicle can develop to a mature stage and can produce hormones enough to support vaginal opening and cornification of vaginal epithelium in ovariectomized mice after 5 days of transplant, and blood vessels also appear in the grafted gel. Mature follicles that are extracted from this grafted gel can be harvested and finally resulted in the embryo through *in vitro* fertilization (55). In the same year, Gosden et al. isolated primordial follicles from infant mice and culture in a plasma clot and then transferred them back into a vacant periovarian capsule which was immediately formed after ovariectomy. All stages of follicular maturation can be seen in the grafted clot, eleven of fifteen mice were pregnancies, and 2 mice produced offspring (56). Dolmans et al. isolated human primordial follicles, embedded in plasma clot, and xenotransplanted to immunodeficient mice. Secondary stage and antral follicles can be found

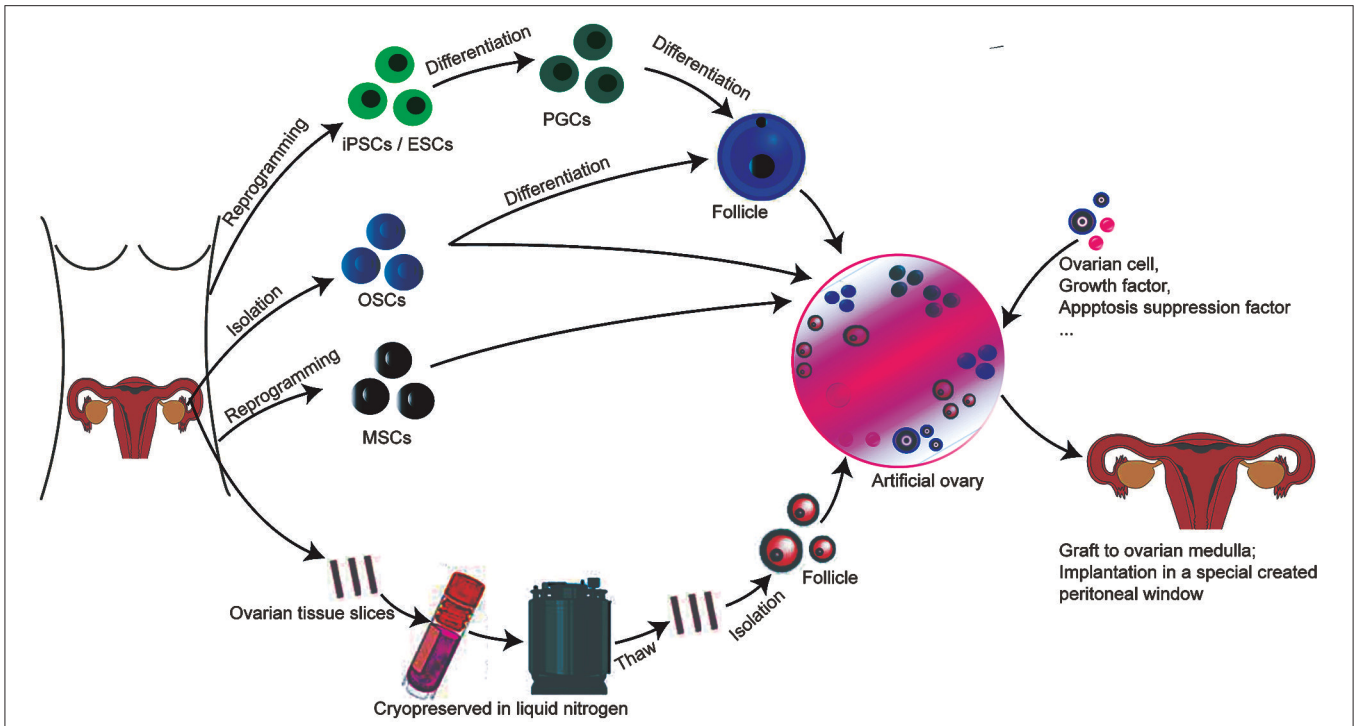

FIGURE 1 | Protocol for constructing an artificial ovary combined with stem cells for patients with BC to preserve fertility and restore endocrine function. (I) If the patient is prepubertal or requires immediate chemotherapy with a potential risk of transmitting malignant cells, ovarian tissue slices are removed and long-term cryopreserved in liquid nitrogen. After thawing, follicles can be isolated from ovarian tissue and then embedded inside a scaffold, we called it "artificial ovary." (II) Patient-specific induced stem cells, such as PGCs, ESCs, and OSCs, can be differentiated to form follicles and embedded into an artificial ovary. OSCs and MSCs can also be directly placed inside the artificial ovary without differentiation. (III) Other additives can also be added into the artificial ovary, such as ovarian cells, growth factors (VEGF, bFGF), and apoptosis suppression factor (S1P). Finally, this transplantable and functional artificial ovary can be grafted to the ovarian medulla or implantation in a specially created peritoneal window.

in clots after 5 months of transplanted, but plasma clots were degraded quickly leading to a large number of follicle losses (57, 58). Hence, the contraction and deformation characteristics of collagen or plasma clot scaffolds are difficult to manipulate, and follicles are easy to lose which has limited their application to load-bearing tissues in the human body (34, 54).

Alginate is a polysaccharide-based natural polymer derived from algae, and the rigidity of alginate can avoid structure from being degraded. Rios et al. encapsulated isolated follicles from mice into alginate matrix and transplanted them back into ovariectomy mice. Many follicles can develop into antral follicles and even mature follicles which can be successfully fertilized by intracytoplasmic sperm injection (ICSI) (59). It is reported that embedded isolated human primordial follicles in alginate gel and culture *in vitro* for 8 days, follicles can develop, and some of them can reach the preantral stage (60). But when the culture *in vitro* for a longer time (>30 days), follicles grow to the antral stage, but many of them were degenerated and stopped growing after further culture (61), since human follicles are larger than mouse follicles, alginate is rigid and cannot be degraded without alginate lyase, which can limit the further growth of follicles and also not conducive for vascularization (62).

Fibrin is another natural polymer to replace plasm colt, fibrinogen and thrombin are the main components of fibrin, and their concentration determines the porosity and hardness of fibrin. Fibrin has high bioadhesion with minimal inflammation after being grafted into the human body and has been widely used for tissue engineering. Paulini et al. isolated human primordial follicles encapsulate in fibrin gel and xenografted in mice, and many of the follicles can grow into the second stage after 7 days of xenografted (63). Long-term (21 days) culture in fibrin gel of isolated mice primordial follicles can also be developed into the antral stage, and hormone levels can be detected in the mice (64). But fibrin has a higher degradation rate in the human body, due to the inherent plasmin and other inhibitors in the human body, follicles will lose the support of the architecture after the degradation. Fortunately, the degradation of fibrin is safe from toxic and can be naturally cleared by the human body (65). In the natural ovary, the outer cortex is more solid whereas the medulla is soft which can allow follicles to migrate from solid cortex to soft medulla (66). An interpenetrating network composed of fibrin-alginate was investigated for embedding mice secondly follicle for short-term culture and produced a higher meiotic rate of oocyte than alginate or fibrin along (67). Longer-term (30 days) cultures of isolated caprine follicles in a fibrin-alginate matrix have a

higher maturation rate than alginate only (68). We can infer that a partially degraded fibrin-alginate matrix is beneficial for follicle survival and proliferation with adjustable rigidity and degradability.

Decellularized ovarian extracellular matrix is another natural matrix, obtained by removing the cellular components from the natural ovary, which can highly mimic the natural ovary *in vivo* allow cells to adhere and grow. Decellularized tissue has been tested in the liver (69), lung (70), and heart (71). Nevertheless, xenotransplantation may induce the immune reaction in the human body and should be paying more attention to further clinical application. Laronda et al. seeded isolated mice follicles into decellularized bovine ovary scaffolds and grafted them to normal ovariectomy mice with normal immune function. After 2 weeks of transplantation, an antral follicle can be discovered in grafted scaffold (38). Isolated mice follicles were also cultured in a decellularized porcine ovary and regrafted back natural pregnancy, and healthy offspring was generated in the POF mouse model after 100 days of graft (72). Hassanpour et al. decellularized the human ovary embedded with isolated rat follicles and grated back into a rat. Hormone and primordial or primary follicle-like structures were detected in this suitable cytocompatibility scaffold after 4 weeks of surgery (73). Pors et al. also successfully embedded isolated human follicles in a decellularized human scaffold and grated them back into a rat for 3 weeks (74). But xenogeneic scaffolds may induce a high risk of the immune response and also may induce some diseases, for example, viruses, or cell residues from the donor (75).

Synthetic polymer has its advantage compared to the natural polymer. It can tailor according to the different hardness of the natural ovary and meet the different clinical requirements (76). Polyethylene glycol (PEG) is widely used for engineering, and oxygen and carbon are the main components of PEG. Kim et al. use PEG hydrogels for embedding isolated mice follicles and grafted into ovariectomized mice, and each stage of follicles and corpora lutea can be discovered in scaffold after 30 days of grafting. Hormone levels improve significantly after 60 days of graft, and functioning blood vessels can also be detected in the scaffold (77). However, the degradation of PEG hydrogels is toxic, and the degradation products can easily cause immune response (78).

3D bioprinting can precisely adjust the pore size and thickness of the stent and can also control the rigid and other properties of the scaffold to meet the clinical needs. It can create the scaffold layer-by-layer to generate a tissue mimic structure (79). Laronda et al. use gelatin as 3D ink to print a scaffold crosslink with 250 μm diameter of the strut, 350 μm diameter of the pore. After seeding isolated mice follicles in 3D printed scaffold, the scaffold was grafted into ovariectomized mice and became vascularized after 7 days of implantation without additional exogenous angiogenic factors. Mature follicles can be found after 3 weeks of implantation, after 10 weeks, these grafted mice were mated, and each recipient mice have one or two litters (80). Other seeding isolated porcine follicles in a scaffold composed of gelatin together with poly(epsilon-caprolactone) (PCL), to construct a structure with 300 μm of pore size and 1 μm diameter of struts. After 10 days of *in vitro* culture, the follicle can adhere well to the stent with good development and a high survival rate (81).

ADDITIVES FOR TRANSPLANTABLE AND FUNCTIONAL ARTIFICIAL OVARY

Stem Cells for Generating New Oocytes in Artificial Ovary

Stem cell is an alternative source and promising strategy for constructing an artificial ovary with regenerative function. Pluripotent stem cells have self-renewal and differentiation functions. Additionally, a functional oocyte in mammals needs multiple steps of generation from a germ cell. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can be induced to primordial germ cells (PGCs) or primordial germ cell-like cells (PGCLCs), in turn, differentiated to the oocyte. Female oogonial stem cells (OSCs) originate from very small embryonic-like (VSEL) stem cells that exist in the ovary and have the ability of oogenesis without inducing differentiation. In oogenesis, these differentiations becoming primary oocytes were also regulated by the environment in the artificial ovary on transplantation into the body.

Induced Pluripotent Stem Cells and Embryonic Stem Cells

Hayashi et al. induced iPSCs to perform PGCLCs and transplanted the PGCLCs into mice seminiferous tubules. After 10 weeks of transplantation, spermatogenesis was exhibited and can form an embryo followed by ICSI and successfully resulted in offspring (82). Subsequently, they derived female ESCs like ESCs and iPSCs to perform epiblast-like cells (EpiLCs) and further induce it to PGCLCs, later coculture it with embryonic gonadal somatic cells to form ovary *in vitro* and then transfer this artificial ovary to mice for oogenesis, follicles at GV stage were detected in 32 days after transplantation, and mature oocyte can be isolated at 53 days after transplantation, which can be well fertilized and generate offspring (83). Hence, ESCs–iPSCs can be a promising source for generating new oocytes for artificial ovaries. Despite these encouraging results, induced iPSCs may have a risk of mitochondrial mutations, and we should pay more attention to pathogenic mitochondrial DNA modifications after transplanting it *in vivo* (84).

Female oogonial stem cells that are extracted on the surface of the ovary can generate primordial follicles. Many studies have confirmed the existence of OSCs in the human ovarian cortex (85, 86). Compared to ESCs and iPSCs, OSCs initially arise from VSEL stem cells and have the ability of oogenesis without inducing differentiation (87). White et al. obtained OSCs from human ovaries and *in vitro* manipulation, and oocytes were generated after 2 weeks of xenotransplantation in mice (88). Zou et al. isolated OSCs from mice ovary and transplanted them into the ovary of POF mice which was induced by chemotherapy. Oocytes were detected in the recipient ovary after 8 weeks of transplantation, and offspring was generated after long-term transplanted (more than 15 weeks) (89). Another repeated study obtained OSCs from mice ovaries and grafted them into adult

mouse intraovarian. Follicles can be successfully generated, and 15% of offspring was delivered after natural mating in these grafted mice (90).

Mesenchymal stem–stromal cells (MSCs) that were obtained from the bone marrow have self-renewal potential without pluripotent function. It also can be retrieved from menstrual blood, cord blood, and adipose tissue as a paracrine provider to support stem cell growth and differentiation (91). Although it cannot directly differentiate to the oocyte, transplantation MSCs can secrete cytokines, signal, and growth factors to promote stem cells such as ESCs, iPSCs, and OSCs in artificial ovary differentiate into oocytes (92). MSCs can also simultaneously support nutrition and immune regulation for the ovary (93). It was reported that transplantation of MSCs can provide nearly 109 cytokines in the ovary to help recover follicles in POF patients (94). Wang et al. grafted green fluorescence protein (GFP, Genechem, China)-positive MSCs to the ovary and found that they gather in the interstitium instead of follicles in the grafted ovary (95). The umbilical cord (UC) is the most promising source of MSCs in humans (UC-MSCs) due to its low oncogenicity and rapid self-renewal. Yang et al. embedded human UC-MSCs into a collagen matrix and then transplanted it into POF mice. After 2 weeks of transplantation, hormone levels and follicles' number have risen significantly, and granulosa cell proliferation and ovarian angiogenesis were detected in the graft (96). Transplantation of MSCs can boost ovarian function and improve the success rate and outcome of the artificial ovary *in vivo*.

Ovarian Cell for Functional Artificial Ovary

An ovarian cell can support angiogenesis, and signal transduction of the artificial ovary is fundamental for follicle proliferation and maturation (97). Ovarian cells can secrete factors that can regulate the transformation of primordial follicles into primary follicles and simulate the microenvironment for follicle growth and survival proliferation (98). There is a positive correlation between the number of human ovarian stromal cells and endothelial cells, the area of angiogenesis, and the survival of follicles in the artificial ovary after transplantation *in vivo* (99). Dath et al. embedded isolated human stromal and endothelial cells together with follicles into plasma clots and xenografting in mice ovary. Fully vascularized stromal structure and higher scaffold degradation were detected in graft after short-term xenograft (100). Additionally, the best source for ovarian cells comes from fresh medulla part in the ovary after cancer remission, and this strategy not only can reduce the risk of reintroducing the malignant to the body, but it also can avoid the damaging effect of cryopreservation to ovarian cells. Because ovarian cells are sensitive to cryopreservation, chemotherapy has less effect on ovarian cells (97). Another source for the ovarian cell is the stem cell. Park et al. isolated stem cells from mice skin, and we can call it skin-derived stem cells (SSCs), induced SSCs differentiation to ovarian-cell-like cells, embedded in Matrigel scaffold, and then transplanted into ovariectomized mice. Estrus cycles were recovered, and follicles and blood vessels were found in the transplants after 8 weeks of transplantation (101).

Factors for Supporting the Artificial Ovary

Growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) can promote angiogenesis and decrease apoptosis for artificial ovary *in vivo*. Shikanov et al. embedded ovarian tissue together with VEGF in fibrin gel and grafted it back into bilateral ovariectomy mice. After 2 weeks of transplantation, a gel containing VEGF has two times as many survival follicles and blood vessels as the control group (102). Another study also encapsulated ovarian tissue together with bFGF in fibrin gel and then grafted it under the skin of mice. After 7 days of transplantation, the bFGF group has higher follicle survival and proliferation rate, lower follicle and ovarian cell apoptosis rate, and higher angiogenesis rate compared to the non-bFGF group (103).

Apoptosis suppression factor sphingosine-1-phosphate (S1P) is one of the apoptosis suppression factors that can induce cell survival and proliferation. It is a signaling sphingolipid that can act as an intracellular second messenger and extracellular ligand for G protein-coupled receptors. It also can regulate angiogenesis and vascular stability (104). Soleimani et al. reported that xenograft of human ovarian into severe combined immunodeficient (SCID) mice together with S1P. After 10 days of transplantation, vascular density, angiogenic, and proliferation of ovarian cells were increased significantly in graft, with lower follicle apoptotic compared to the control group (104). Another research embedded follicles together with S1P and VEGF into fibrin scaffold and generated two times as many primordial follicles, blood vessels, and offspring compared to the control group (105).

CONCLUSION AND FUTURE ENDEAVORS

The number of young women who are diagnosed with BC has risen continuously in recent years. Simultaneously, the development of modern therapeutic significantly improved the survival rates and prolong the life expectancy. Hence, fertility preservation turned out to be an urgent request for young females before gonadotoxic therapy. Artificial ovary combined with stem cell can mimic natural ovary as a promising strategy for patients with BC that meets the needs of recover fertility and restore gonadal hormone function without reintroducing the malignant cells and delaying their cancer therapy.

As a transplantable in the human body, the scaffold of artificial ovary should allow follicle survival and proliferation, facilitate the formation of blood vessels and stroma *in vivo*, and should be safe for the human body. Although animal research has generated few offspring on artificial ovaries, more experiments and animal studies should be tested to search for a suitable scaffold for transplantable artificial ovaries. Due to the finite source of follicles, stem cells as an alternative to female gametes bring great hope for future clinical implementation. Yet, stem cell therapy is still in the research stage and is insufficient for clinical use. More research is needed to verify and test for fill gaps that may lead to clinical benefits in the future.

AUTHOR CONTRIBUTIONS

JC wrote the manuscript and figures. JC, LR, and YL reviewed the literature. QC, XY, and WS conceived the framework of this review article, provided insights, and edited the manuscript. VI and HH revised and polished the manuscript. UK and JH edited the grammar and revised the manuscript. YL polished the final manuscript and figure. All authors contributed to the article and approved the submitted version.

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Analysis of Aneuploidy Rate and Pregnancy Outcomes in Unexplained Recurrent Pregnancy Loss Couples With Chromosome Polymorphism After PGT-A

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Purpose: The study aims to investigate whether chromosomal polymorphism affects embryo development and pregnancy outcomes of unexplained recurrent pregnancy loss (uRPL) couples undergoing PGT-A.

Methods: A total of 585 couples with uRPL history who performed PGT-A were included in the retrospective study from January 2016 to December 2020. We included 415 couples with normal karyotype and 170 couples with chromosomal polymorphism. Furthermore, the polymorphism group was divided into two subgroups: 113 couples in the male group and 57 couples in the female group. The embryo development and pregnancy outcomes were analyzed in different groups.

Results: The blastocyst rate and aneuploidy rate are statistically different in the normal group, male polymorphism group, and female polymorphism group. Compared with normal and female groups, the male group has a lower blastocyst rate, which is statistically different (48.3 vs. 53.9%, $p = 0.003$; 48.3 vs. 54.1%, $p = 0.043$). Moreover, the aneuploidy rate of the male polymorphism group is significantly higher than female carriers (29.5 vs. 18.6%, $p = 0.003$). However, there were no statistically significant differences in clinical pregnancy rate, early miscarriage rate, and live birth rate after PGT-A ($p > 0.05$).

Conclusion: Male with chromosome polymorphism (CPM) have a lower blastocyst rate and a higher aneuploidy rate than female carriers in uRPL couples undergoing PGT-A. However, when a euploid blastocyst was first transferred, no difference in pregnancy outcomes was found between the male and female polymorphism carriers. It indicated that CPM may have an adverse effect on the embryos of male carriers with uRPL history, and the occurrence of uRPL may be decreased in male polymorphism carriers after PGT-A.

Keywords: chromosome polymorphism, aneuploid, recurrent pregnancy loss, preimplantation genetic testing, gender

INTRODUCTION

Chromosomal polymorphism is considered as quantitative or positional alterations in constitutive DNA heterochromatin, often occurring in the centromeric region of chromosomes 1, 9, 16, Y, and short arms of acrocentric chromosomes (ACRs), such as those in D and G groups (chromosomes 13, 14, 15, 21, and 22). Studies reported that it played an important role in spindle attachment, chromosome movement, meiotic pairing, and sister chromatid cohesion. However, the real impact of chromosomal polymorphism in the human genetics remained controversial. Some studies have shown that chromosome polymorphism (CPM) was a normal chromosome karyotype with no related phenotypic and functional effects, whereas others think that CPM may have a certain impact on infertility people and recurrent pregnancy loss (RPL) (1–4).

At present, some studies reported a close association between chromosomal polymorphisms and unexplained infertility couples with reproductive disorders. The karyotype of polymorphism accounted highly in infertilities. Cheng et al. reported that the incidence of polymorphism variants in the infertile population was higher than in fertile patients (5.53 vs. 3.74%) (3). Moreover, in other studies, the patients who have experienced RPL were found to have a higher frequency of chromosomal polymorphisms (8–15%) in comparison with patients of other infertility causes and natural pregnancy (3–10%) (4–6). Therefore, RPL may be related to CPMs based on the above studies.

There are many studies with conflicting points on whether polymorphism causes an adverse impact on the pregnancy outcomes in IVF treatments. According to the study of Hong et al., chromosomal polymorphism seemed to have no adverse effects on pregnancy outcomes of IVF-ET treatment. They found no differences between the polymorphism groups and the control group in the rates of implantation and clinical pregnancy after IVF/ICSI treatment (7). However, others reported that the rates of spontaneous miscarriage and preterm birth in infertility women with CPM were significantly higher than with normal karyotype (3).

In addition, many studies that investigated the gender factor of polymorphic carriers had reported that chromosomal polymorphism occurred more frequently in male partners than female partners within recurrent spontaneous abortion couples, which mainly involve the Y chromosome. It might lead to impaired sperm quality and quantity (4, 5, 8, 9). Ni et al. reported that the first embryo transferred rate and cumulative live birth rate of male polymorphism carriers were significantly lower than those of female carriers and normal karyotype couples in IVF/ICSI treatments (10). It may prove a connection between gender and CPMs. Therefore, we conducted further research on the embryo quality and pregnancy outcome after preimplantation genetic testing (PGT) treatment, to estimate whether chromosomal polymorphism has an impact on the outcome of ART.

Most present studies focused mainly on the analysis of IVF/ICSI reproductive outcomes in CPM carriers, instead of PGT. The embryo quality, especially the rate of aneuploid following a single oocyte retrieval, is also the most concerned issue for clinicians and patients, but never analyzed as the main outcome measure in previous chromosomal polymorphism studies. PGT can detect the quality of embryos in couples with chromosomal polymorphism, which can help us judge whether polymorphism affects pregnancy outcomes genetically. Therefore, the aim of our study is to explore whether CPM and carrier's gender affect pregnancy outcomes, and to provide a clinical guidance about the follow-up reproductive outcomes of polymorphic couples in uRPL couples.

MATERIALS AND METHODS

Study Population

The retrospective analysis included 585 couples who have experienced unexplained recurrent pregnancy loss (uRPL) from January 2016 to December 2020 in our center. All couples received at least one cycle of PGT treatment. Female age was from 23 to 45 years old, and basal follicle-stimulating hormone (FSH) was under 10 IU/L. Antral follicle count (AFC) in one ovary was divided into three groups: normal ovarian response (NOR) (AFC5–10), poor ovarian response (POR) (AFC < 5), and polycystic ovary (PCO) (AFC > 10), which was used to assess ovarian reserve.

The definition of RPLs is controversial. RPL is defined as two or more clinical miscarriages confirmed by ultrasound or histology and biochemical pregnancy failures, not necessarily consecutive (ASRM Practice Committee). We included couples who we considered as having uRPL history. So, we excluded pregnancy loss with clear reason who fulfilled the following criteria, to reduce other factors that interfere with embryo quality and pregnancy outcomes (11–13), including (a) abnormal chromosome karyotypes, monogenic diseases, and both male and female were diagnosed as polymorphism carriers; (b) abnormal uterine anatomy assessed by hysteroscopy, hysterosalpingography, or uterine sonography; (c) endocrine diseases, such as hyperprolactinemia, hyperthyroidism, or hypothyroidism; and (d) autoimmune factors, such as antiphospholipid syndrome. Therefore, the above is the standard of uRPL population in our data.

Then, a total of 585 uRPL couples were grouped according to the karyotype and different genders: normal group included 415 couples with normal chromosomes, the male polymorphism group consisted of 113 couples, and the female consisted of 57 couples. Ethical approval for the use and analysis of information and data from patients who underwent PGT-A was obtained from the Ethics Committee of the Center for Reproductive Medicine, Shandong University. Informed consent was obtained from all the patients included in this study.

Study Procedure

The procedure of PGT involves performing a controlled ovarian stimulation cycle, followed by mature oocyte retrieval and ICSI with the partner's sperm. The resulting embryos, usually at the blastocyst stage, are then biopsied. The embryo is then tested for genetic abnormalities, and only the embryos with the normal DNA are later transferred (13). Appropriate ovarian stimulation protocols were given according to the female's age and ovarian

reserve function in our center. These protocols included long and short GnRH agonist, antagonist, and others, like mild stimulation or super-long protocols. The long protocol started with GnRH agonist administrated in the mid-luteal phase of the previous cycle and combined with recombinant FSH when pituitary desensitization was achieved in this cycle. Additionally, the short protocol started with the administration of GnRH agonist and recombinant FSH together on day 2 or 3 of this cycle. The antagonist protocol was used similarly to the short protocol, but it started with a GnRH antagonist. Then, the dosage of recombinant FSH was regulated according to size of the follicle and serum E2 concentration (7). An HCG trigger for final oocyte maturation was implemented when at least two follicles with diameters ≥18 mm were detected, and oocyte retrieval was performed 34–36 h later. For all couples, fertilization was achieved by ICSI. High-quality embryos (D3) were selected according to the Gardner criteria (14). At least one morphological high-quality embryo (D3) was cultured to the blastocyst stage (D5 or D6) for trophectoderm biopsy per retrieval. A total of 2,460 blastocysts were genetically screened, of all blastocysts tested using next-generation sequencing (NGS).

The embryos of D5 or D6 were subsequently frozen after biopsy, and it was suggested that only a single euploid blastocyst can be transferred in adaptable time. Only the first embryo transfer cycle was evaluated. Mosaic embryos were not transferred in our study. The endometrium was prepared by natural ovulation cycles or other artificial cycles, depending on the individual conditions. Luteal-phase support was initiated when the endometrial thickness reached ≥7 mm and continued until 12 weeks of gestation.

The serum hCG levels were measured 14 days after embryo transferred, at which time biochemical pregnancy can be diagnosed if the hCG was ≥25 IU/L. A transvaginal ultrasound scan was performed 7th or 8th week after the embryo was transferred, and clinical pregnancy was diagnosed if an intrauterine gestational sac was observed; otherwise, it was confirmed biochemical pregnancy loss (hCG positive, no ultrasound confirmed). Pregnancy termination before gestational age of 12 weeks was considered as an early miscarriage. Live birth per retrieval was defined as the delivery of a viable infant at ≥28 weeks of gestation after the embryo was transferred.

The primary outcome is development of embryos, such as blastocyst rate and aneuploidy rate. Moreover, the miscarriage rate, clinical pregnancy rate, and live birth rate were regarded as secondary outcomes between the three groups.

Statistical Analysis

One-way ANOVA and independent sample *t*-test were used for continuous variables. The rates and categorical variables were compared by the chi-square test and Fisher's exact test. A value of $p < 0.05$ was considered statistically significant. A multiple linear regression model was also conducted to examine the impact of various factors on embryonic development. Chromosomal polymorphism was regarded as categorical variables were transformed to dummy variables. All the statistical analyses were performed using IBM SPSS version 25.0 software.

RESULTS

The Baseline Characteristics, Embryo Quality, and Pregnancy Outcomes in Three Groups

The characteristics of normal group, male polymorphism group, and female polymorphism group (only one spouse with CPMs)

TABLE 1 | Baseline characteristics of uRPL couples.

Variable	Normal *n* = 415	Male *n* = 113	Female *n* = 57	*p*-value
Female age	34.33 ± 4.61	34.05 ± 4.94	33.58 ± 4.81	0.485
<38	70.8%(294)	72.6%(82)	71.9%(41)	0.932
≥38	29.2%(121)	27.4%(31)	28.1%(16)	
Male age	35.05 ± 4.92	34.65 ± 5.00	33.68 ± 4.77	0.130
BMI	23.83 ± 3.18	23.68 ± 3.66	23.50 ± 2.97	0.736
FSH	6.73 ± 1.84	6.58 ± 1.77	6.93 ± 2.27	0.486
AMH	3.34 ± 2.56	3.21 ± 2.05	3.55 ± 2.77	0.705
AFC				0.631
NOR (5–10)	68.7%(285)	74.3%(84)	64.9%(37)	
POR (<5)	17.6%(73)	15.0%(17)	22.8%(13)	
PCO (<10)	13.7%(57)	10.6%(12)	12.3%(7)	
Ovarian stimulation protocols				0.170*
Long	34.9%(145)	44.2%(50)	31.6%(18)	
Short	26.3%(109)	21.2%(24)	15.8%(9)	
Antagonist	33.5%(139)	31.0%(35)	47.4%(27)	
Others	5.3%(22)	3.5%(4)	5.3%(3)	
No. oocytes obtained	11.52 ± 6.12	11.67 ± 5.62	11.32 ± 6.42	0.935
No. MII oocytes	10.00 ± 5.62	10.15 ± 4.91	10.00 ± 5.82	0.967
Fertilization rate (%)	79.5%(3298/4150)	78.1%(896/1147)	81.1%(462/570)	0.348
Endometrial thickness on hCG day(cm)	0.86 ± 0.17	0.85 ± 0.15	0.90 ± 0.16	0.336

**Fisher's exact test.*

TABLE 2 | The embryo quality and pregnancy outcomes of uRPL couples.

Variable	Normal *n* = 415	Male *n* = 113	Female *n* = 57	P1	P2	P3
Embryo quality						
No. D3 embryos	4.96 ± 3.35	4.83 ± 3.23	5.00 ± 3.67	NS	NS	NS
No. Blastocyst	4.28 ± 3.04	3.83 ± 2.42	4.39 ± 3.26	NS	NS	NS
Blastocyst rate (%)	53.9% (1777/3298)	48.3% (433/896)	54.1% (250/462)	0.003	0.926	0.043
Euploidy rate (%)	47.4% (711/1500)	44.3% (177/400)	51.6% (111/215)	0.262	0.246	0.080
Aneuploidy rate (%)	27.7% (416/1500)	29.5% (118/400)	18.6% (40/215)	0.485	0.005	0.003
Pregnancy outcome						
Biochemical pregnancy loss (%)	10.7% (23/214)	17.9% (10/56)	3.4% (1/29)	0.148	0.326*	0.089*
Clinical pregnancy(%)	67.7% (191/282)	61.3% (46/75)	70.0% (28/40)	0.297	0.773	0.355
Early miscarriage(%)	15.7% (30/191)	13.0% (6/46)	14.3% (4/28)	0.651	1.000*	1.000*
Live birth (%)	35.1% (99/282)	37.3% (28/75)	37.5% (15/40)	0.720	0.767	0.986

*NS, not statistically significant; P1, normal vs. male; P2, normal vs. female; P3, male vs. female. *Fisher's exact test.*

are listed in **Table 1**. In the three groups, no statistically significant differences were observed regarding female age, basal FSH, BMI, AMH, AFC, the number of oocytes obtained and MII oocytes, ovarian stimulation protocols, and endometrial thickness on HCG day ($p > 0.05$).

Moreover, **Table 2** showed that the blastocyst rate and aneuploidy rate were statistically different in the normal group, male polymorphism group, and female polymorphism group. It was found that the blastocyst rate of the male polymorphism group was statistically lower than that of the normal group and female group (48.3 vs. 53.9%, $p = 0.003$; 48.3 vs. 54.1%, $p = 0.043$). More importantly, a phenomenon to higher aneuploidy rate in male polymorphism group than female carriers was noted, which was statistically significant (29.5 vs. 18.6%, $p = 0.003$). Compared with female polymorphism group, the polymorphic karyotype may have a partial effect on embryo development in men. Moreover, when analyzed the pregnancy outcomes after the first euploid embryo transplantation in three groups, the biochemical pregnancy loss rate, early miscarriage rate, clinical pregnancy rate, and live birth rate had no statistical difference ($p > 0.05$). **Figure 1** also clearly showed that, male polymorphism carriers had a low blastocyst rate and a high aneuploidy rate, but there was no significant difference in pregnancy outcomes with the normal group and the female group. It probably indicated that the embryo quality of male polymorphic carriers is worse than that of female, and PGT could influence the pregnancy outcomes.

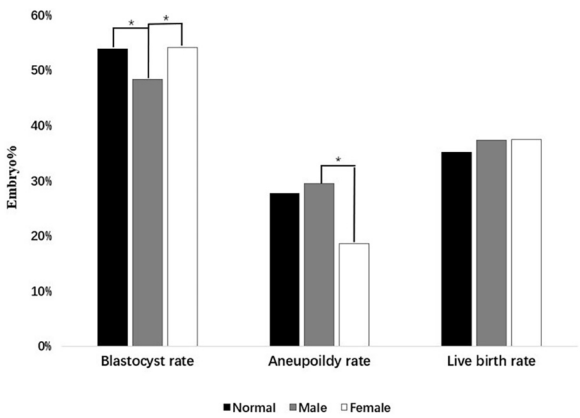

FIGURE 1 | The embryo quality between three groups. The difference in Blastocyst rate, Aneuploidy rate, and Live birth rate are shown in this figure. The symbol "*" indicates a significant statistical difference ($p < 0.05$).

Prevalence of Different Types of Chromosomal Polymorphisms

The incidence and types of polymorphism in 170 uRPL couples with CPM are shown in **Table 3**. A total of 178 chromosomal polymorphisms occurred in 170 couples, with a frequency of 119 for the male polymorphism group and 59 for female polymorphism group. Only one person in each couple was a polymorphic carrier. There were six men with ≥2 CPMs in the male polymorphism group and two women in the female polymorphism group. The type of ACR accounts for a higher proportion (30.3%) in all kinds of chromosomal polymorphism, in which chromosome 21 was the majority. The abnormality of chromosome 21 was also a common cause of miscarriage. Moreover, the most common polymorphisms observed were Y chromosome variants (31.1%) in the male group, especially Yqh+, whereas 1qh+ was the most in female group. Whether in male or female polymorphism groups, 1qh+ had a large proportion in type qh+. It indicated that the types of CPM were slightly different in different genders. In **Figure 2**, the abnormal chromosome 16 had the highest proportion in the aneuploidy embryos of polymorphism group, followed by chromosomes 22 and 1. Furthermore, we analyzed the influence of parental

TABLE 3 | Frequency of chromosomal polymorphism variation.

Types	Total	Male (*n* = 113)	Female (*n* = 57)
qh	49(27.6%)	27(22.7%)	22(37.3%)
1qh	43	23	20
9qh	3	3	0
16qh	3	1	2
ACR	54(30.3%)	30(25.2%)	24(40.7%)
13ps(s)/pstk(stk)	7	5	2
14ps(s)/pstk(stk)	7	4	3
15ps(s)/pstk(stk)	15	7	8
21ps(s)/pstk(stk)	18	10	8
22ps(s)/pstk(stk)	7	4	3
Inv(9)	38(21.3%)	25(21.0%)	13(22.0%)
Y-chromosome	37(20.8%)	37(31.1%)	–
Inv(Y)	4	4	–
Yqh–	14	14	–
Yqh+	19	19	–
Total	178	119	59

ACR, acrocentric chromosome.

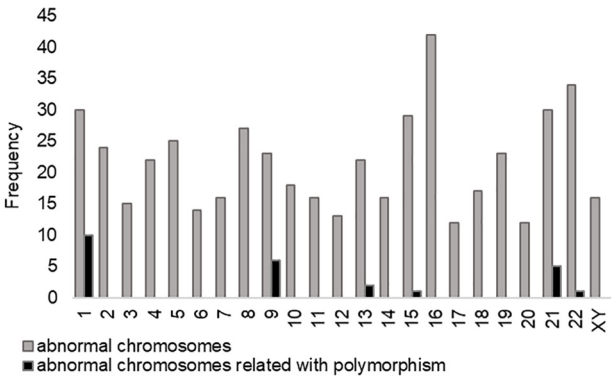

FIGURE 2 | The frequency of abnormal chromosomes in the aneuploidy embryos of polymorphism group. Chromosome 16 has the highest abnormal proportion in aneuploidy embryos, followed by chromosomes 22 and 1. The proportion of abnormal chromosomes associated with chromosome polymorphism (CPM) accounts for 5.04% (25/496) in all abnormal chromosomes, and chromosomes 1, 9, and 21 appear more frequently among related chromosomes.

CPM in the karyotype of aneuploidy embryos (black column), to estimate whether the chromosomal polymorphisms are inherited after PGT treatments. The proportion of abnormal chromosomes associated with parental CPM accounts for 5.04% (25/496) in all abnormal chromosomes, and chromosomes 1, 9, and 21 appear more frequently among related chromosomes. It showed that the karyotype of embryos may have some correlations with parental CPM.

Multiple Linear Regression on Embryo Quality of 585 uRPL Couples

As shown in **Table 4**, a multiple linear regression model was conducted to comprehensively evaluate the impact of female age, female FSH, BMI, oocytes obtained, and the polymorphic carriers' gender on the number of blastocyst and aneuploidy. It was obvious that female age, BMI, and oocytes obtained have a significant effect on blastocyst and aneuploidy ($p < 0.05$), whereas FSH does not. According to the analysis of linear regression, the number of blastocysts in the male polymorphism group was significantly lower than the normal group (β = –0.071, 95%CI –1.006 to –0.064, $p = 0.026$). It showed that male CPM may have some negative effects on embryo development.

DISCUSSION

The previous studies have reported that the detection rate of CPM in the infertilities is higher than in the fertilities, and the overall incidence of CPM increases in infertile patients in recent years. Our study shows that the types of 1qh+, inv (9), and 21ps(s)/pstk(stk) were prevalent in uRPL people with polymorphic variations, and the incidence of chromosomal polymorphisms in men was found to be higher than that in women. The frequency of polymorphic variation on chromosome 1 is high in both male and female carriers, particularly in women (15). Moreover, chromosome 1 is also the largest one in the human chromosome group and has rich genetic material, and many genetic diseases are related to it. The abnormalities of chromosome 21 in embryos are one of the most common causes of RPL in elderly women, such as trisomy 21. The polymorphism of chromosome 9 is often thought to be associated with the meiosis of gametes. Moreover, the conclusions of some studies are slightly different from us, and they reported that chromosome 9 was the most common polymorphic variation in infertile couples, such as inv (9) and 9qh+, but not too much about the abnormalities on chromosome 1 or 22 (2, 3, 7).

Cheng et.al subdivided the female infertility group based on the reason of infertility to explore the association between CPMs and female infertility. It was found that unexplained infertility accounted for the largest proportion in CPM people than fallopian tube infertility, ovulation disorder infertility, and uterine infertility (3). Therefore, it was reasonable to suspect that unexplained infertility may be related to CPM. At the same time, the adverse pregnancy outcomes of female polymorphism carriers have also increased than normal female polymorphism carriers after excluding male polymorphism carriers. Among these pregnancy outcomes, the miscarriage rate of polymorphic female is significantly higher than that of normal karyotype female in the cause of fallopian tube infertility (6.17 vs. 1.08%). In addition, other studies have found that there was no clinical meaning when merely studying the influence of CPM on embryo quality, but the genetic effects may be presented when polymorphism coexists with other chromosomal abnormalities. For example, patients with both polymorphism and translocation (CTCPM group) have a lower rate of high-quality embryos and a higher rate of abnormal embryos than the patient with simple translocation (CT group) ($p < 0.05$). The rate of high-quality embryos was also lower in men than women in the CTCPM group (16). Therefore, we believed that

TABLE 4 | Multiple linear regression for the number of blastocyst and aneuploidy in uRPL couples.

Variable	No. blastocyst			No. aneuploidy		
	β	95%CI	*p*	β	95%CI	*p*
Female age	–0.162	–0.143, –0.061	<0.001	0.430	0.084, 0.121	<0.001
FSH	–0.014	–0.128, 0.083	0.675	–0.072	–0.090, 0.004	0.076
BMI	–0.078	–0.128, –0.014	0.015	–0.084	–0.054, –0.003	0.028
Oocytes obtained	0.569	0.245,0.311	<0.001	0.202	0.022, 0.052	<0.001
Polymorphism						
Male[a]	–0.071	–1.006, –0.064	0.026	0.021	–0.153,0.272	0.582
Female[a]	0.007	–0.563, 0.693	0.839	–0.058	–0.499, 0.065	0.131

[a]*Male group or female group compared with normal group.*

the compound mutation of chromosomes polymorphism may affect the patient's embryo quality and molecular karyotype in the above studies.

Moreover, a study reported that cooccurrences of different types of CPMs probably affect patients' clinical phenotype, such as IVF failure, infertility, and recurrent miscarriage. It indicated that there may be an interchromosomal effect between chromosomes with polymorphism and other chromosomes (17). Others reported that chromosomal polymorphisms were associated with an increase in the occurrence risk of multinucleated embryos in the IVF/ICSI cycle. There occurs the incidence of CPM and miscarriage rate in multinucleated embryo group than that of the control group. More importantly, the phenomenon was more significant in men, but not in women (18). To sum up, clinical phenotypes may occur when CPMs and other factors work together, especially the gender factor. Similar results were presented in our study, and we found that the effect of CPM on pregnancy outcomes was related to the gender of polymorphism carriers after excluding those with complex chromosomal mutations and definite infertility reasons in our study. The embryo quality of male polymorphism carriers was worse than female carriers.

In Wilson et al.'s study, the incidence of long heterochromatic polymorphism variants in infants undergoing IVF, ICSI, and natural pregnancy was compared. It was found that infertile couples who obtained pregnancy through ART were not more likely to inherit chromosomal polymorphism than those through natural pregnancy (19). There is no significant difference in the detection rate of infant chromosomal polymorphisms, which may be because the embryos affected by CPM are naturally eliminated when embryo development or the CPMs may have no genetic effects on embryo development and pregnancy outcomes. Our study found a total of 496 abnormal chromosomes in the aneuploidy embryos of chromosome polymorphic couples, of which 25 chromosomes were associated with couples' chromosomal polymorphism (**Figure 2**). The proportion accounts for 5.04% (25/496), and chromosomes 1, 9, and 21 appear more frequently among related chromosomes. It is also consistent with the occurrence of CPM as shown in **Table 3**. As a result, embryos are less likely to have the same chromosomal abnormalities as their parents, and the heritability of chromosomal polymorphisms is poor.

The effects of chromosomal polymorphisms on IVF/ICSI treatment are controversial as well. Some studies think that chromosomal polymorphisms had no apparent adverse pregnancy outcomes on IVF treatment. Hong et al. investigated the pregnancy outcomes after IVF treatment in male, female, and normal groups manifesting as no difference. Others reported that CPM affects the pregnancy outcomes (7). A recent study found that CPM in either male or female carriers seemed to have adverse effects on IVF/ICSI outcomes (15). Liang et al. reported that a significantly lower fertilization rate was found in infertility male compared with female and normal karyotypes (20). When stratified according to the fertilization method, the use of ICSI could increase the fertilization rate for men with chromosomal polymorphisms than IVF. Male carriers affected outcomes by decreasing the rates of fertilization, good quality embryos, clinical pregnancy, and live birth as well as increasing the biochemical pregnancy rate ($p < 0.05$), while in female carriers only by decreasing the embryo cleavage rate ($p < 0.05$). Notably, many studies included cohorts of mixed IVF or ICSI treatment. However, all cases underwent ICSI as an insemination method in our study. Moreover, a meta-analysis concluded that male chromosomal polymorphism showed lowered values for fertilization rate, cleavage rate, good quality embryos rate, and live birth rate. However, no similar correlation was found in female chromosomal polymorphism (21). The results obtained in our study could be an explanation for the results found by Ni et al. (10), it reported that male polymorphism carriers have a lower live birth rate per transfer cycle than women after IVF/ICSI treatment, and the early miscarriage rate has a rising trend ($p < 0.05$). Similar results were presented in our study, and we found there was no significant difference in pregnancy outcomes between male polymorphism carriers, female polymorphism carriers, and normal karyotype, but the aneuploidy rate of men with CPM is significantly higher than women. We speculated that this difference was mainly due to that all the transferred embryos were screened by PGT-A treatment. PGT exerted selection pressure toward the embryos to be implanted. It was probably that PGT-A could increase the euploid transferred rate and live birth rate in male polymorphism carriers compared with

the couples who performed IVF/ICSI treatment. Therefore, men with CPMs in uRPL couples are more suggested to perform PGT, which decreases the risk of couples with recurrent miscarriages and reduces physical and psychological damage to women.

Morales et al. (22) revealed that CPM may have an impact on male fertility. The study analyzed the relationship between CPM and the aneuploidies in male gametes and embryos. As has been observed in the previous studies, men with CPM have an increased rate of sperm aneuploidy compared with normal men. We noted related results to Morales et al's study. Male polymorphism carriers have a higher aneuploidy rate than female groups in uRPL couples and have generally lower rates of blastocyst and euploidy than female. The possible explanation for the above phenomenon may be the heterochromatin that plays an essential role in meiosis. Chromosomal polymorphism may impair the formation of functional gametes. Consequently, patients who are polymorphism carriers might theoretically be more susceptible to having an increased incidence of embryonic aneuploidy and impaired reproductive outcome (22). Certain biological effects of CPM could have sex-specific on cell division, particularly in the Y chromosome. In many recent studies, the variation of Y chromosome may increase the rate of errors in meiotic segregation and recombination. Some found lower fertilization rates in CPM carriers with severe oligozoospermia, compared with non-carriers with severe oligozoospermia (23). Thus, it suggested that polymorphism might have adverse effects on spermatogenesis and a negative impact on IVF outcomes.

Regarding RPL, the definition of it was the loss of two or more pregnancies before 20 weeks of gestation. It contains non-visualized pregnancy losses that combine biochemical pregnancy loss (positive hCG, no ultrasound performed) and failed PUL (positive hCG, but no pregnancy location established) (11, 12). Excluding the common causes, such as uterine abnormalities, hormonal disorders, infections, and cytogenetic abnormalities, more than 40–50% of RPL patients have no clear reason. It was considered as uRPL. So, the research to explore additional etiologies for uRPL is critically important (13, 24). Some studies reported that couples with RPL history have a significantly higher rate of sperm DNA fragmentation and a lower proportion of spermatozoa with normal morphology compared to fertile control women (8, 9). In addition, some reported that the sperm aneuploidy and DNA fragments had probably been associated with abnormal meiotic recombination. The high incidence of polymorphism in male might support the opinion that the polymorphism affects the chromosomal pairing and leads to meiotic arrest (5, 25). Therefore, sperm DNA fragmentation may be an underlying pathogenesis in uRPL people. There is a certain connection among male polymorphism variants, sperm DNA fragments, and RPL history.

Our study has some research values, but also many limitations. To our knowledge, there is currently no comprehensive investigation to report the impact of chromosomal polymorphism in uRPL couples on embryo development after PGT. Furthermore, we only included couples with uRPL after formulating the exclusion criteria, and our results may not apply to other groups of patients. The difference was significant for men and women in our study, perhaps because of the inadequacy of sample size for women. So, these results needed to be confirmed with additional studies in larger populations. Moreover, well-powered prospective studies in the number of CPMs and pregnancy outcomes are needed to fully evaluate whether polymorphism has clinical effects.

CONCLUSION

The major findings of this study are that the embryo quality of male and female polymorphic groups is different in uRPL patients, male carriers with chromosomal polymorphism have a lower blastocyst rate and a higher aneuploidy rate than female carriers, but the pregnancy outcome has no difference. So, it also reminds us that the synergy of CPM and gender contribute to embryo quality. Screening the embryos may be a good option for the male polymorphic carriers with uRPL history. Preimplantation genetic testing (PGT) provides some fertility guidance for uRPL couples and reduces the occurrence of uRPL, particularly in male.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JY conceived and designed the study. MC analyzed the data and drafted the manuscript. QZ and WZ collected and verified the data. YZ and HL revised the manuscript. All authors contributed to conception and design of the study and were involved in interpreting the data and approved the final manuscript.

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Maternal and Neonatal Outcomes After Assisted Reproductive Technology: A Retrospective Cohort Study in China

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Background: With the progress of assisted reproductive technology (ART) and the increasing number of ART pregnancy, its safety has become the focus of attention. The present study aimed to explore the associations of ART pregnancy with maternal and neonatal outcomes, as compared with naturally pregnancy.

Methods: This retrospective cohort study included all pregnant women who delivered at Women's Hospital of Nanjing Medical University in 2011–2020. We compared maternal characteristics and pregnancy outcomes between group of ART pregnancy and group of naturally pregnancy using Logistic regression adjusted for confounders.

Results: A total of 13,604 ART pregnancies and 198,002 naturally pregnancies were included. The proportion of ART pregnancies has increased every year for the past 10 years, peaking in 2020 (9.0%). Multivariable logistic regression analysis showed that the risks of gestational diabetes, preeclampsia, moderate or severe anemia, liver-related diseases, thyroid-related diseases, preterm birth, placenta previa, postpartum hemorrhage, and cesarean section were significantly increased in ART pregnancy. For neonatal outcomes, women conceived by ART were more likely to have twins or multiples, and the risk of stillbirth or abnormal development was also significantly increased. When restriction to singletons, these risks were reduced. And the effects of ART on the risk of premature rupture of membrane, cord entanglement, intrapartum fever, cesarean section, and stillbirth or abnormal development were more pronounced in singletons pregnancies compared with that in pregnancies of twins or multiples.

Conclusion: Women conceived by ART were at increased risks of several adverse pregnancy outcomes compared with women conceived naturally. Multiple pregnancies could partly explain this phenomenon. For ART pregnancy, prenatal and intrapartum monitoring should be strengthened, and neonatal outcomes should be closely observed.

Keywords: maternal outcomes, neonatal outcomes, art, cohort, China

INTRODUCTION

Since the birth of the first test-tube baby in 1978, assisted reproductive technology (ART) has become an effective treatment for infertility (1). With the progress of technology and provision of services, an increasing number of infants are born following ART therapy (1, 2). In developed countries, ART pregnancies account for 1.5–5.9% of all births (3–7), while in China, ART pregnancies account for 1.7% and are increasing year by year (2). ART is sometimes defined differently and is usually defined as the application of laboratory or clinical techniques to gametes and/or embryos for reproductive purposes, however it has been broadened to include not only *in vitro* procedures but also ovarian stimulation with gonadotropins or ovariotropic drugs (8, 9). As these and other reproductive technologies expand, leading to a substantial number of successful pregnancies and births, it is critical for prospective parents to understand the maternal and neonatal outcomes associated with ART.

Several studies have shown that ART pregnancies have an increased risk of multiple pregnancy and adverse pregnancy outcomes, including gestational diabetes, gestational hypertension, placenta previa, preterm birth, operative delivery, low birth weight, birth defects and perinatal mortality (10–16). However, other studies have concluded ART pregnancies do not have increased risks of adverse perinatal outcomes (7, 17–19). The incidences of small for gestational age, preterm birth and cesarean section are similar between ART and naturally pregnancies (13, 20). Nevertheless, pregnancy outcomes in ART pregnancies appear to be generally poorer due to the increased risk of multiple pregnancies. Multiple pregnancy is a post-processing confounding factor, which appears after ART treatment and may confound causal effects. Many previous studies did not adjust for maternal age, BMI and other confounding factors (12, 21–23). It is not clear whether the increased risk of adverse pregnancy outcomes is due to ART itself, multiple births, or potential infertility. At present, opinions are too far apart to reach a consensus.

The present retrospective cohort study was conducted to compare maternal and neonatal outcomes between ART and naturally pregnancies, and in addition to explore the association of ART with adverse pregnancy outcomes by stratifying on birth plurality and maternal age.

Abbreviations: ART, assisted reproductive technology; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; OI, ovulation induction; GIFT, gamete intra-fallopian transfer; AI, artificial insemination; HIS, hospital information system; NLP, natural language processing; BMI, body mass index; OR, odds ratio; CI, confidence intervals.

MATERIALS AND METHODS

Study Design and Population

This retrospective cohort study included all pregnant women who delivered at Women's Hospital of Nanjing Medical University in 2011–2020. The Women's Hospital of Nanjing Medical University is the largest maternity hospital in Jiangsu province, China and delivers approximately 20,000 babies annually. After excluding women who had early abortions (≤12 weeks), or women who were discharged from care during pregnancy, a total of 211,606 pregnancies were included in the data analysis. Two cohorts were created: women who conceived by either intracytoplasmic sperm injection (ICSI), *in vitro* fertilization (IVF), ovulation induction (OI), gamete intra-fallopian transfer (GIFT), or artificial insemination (AI), were defined as group of ART pregnancy, and women who conceived naturally without ART, were considered as group of naturally pregnancy.

Data Collection

We obtained all maternal and neonatal information from Hospital Information System (HIS) Database. Data were collected from standardized clinical forms and hospital records after maternity discharge to form the research database. All

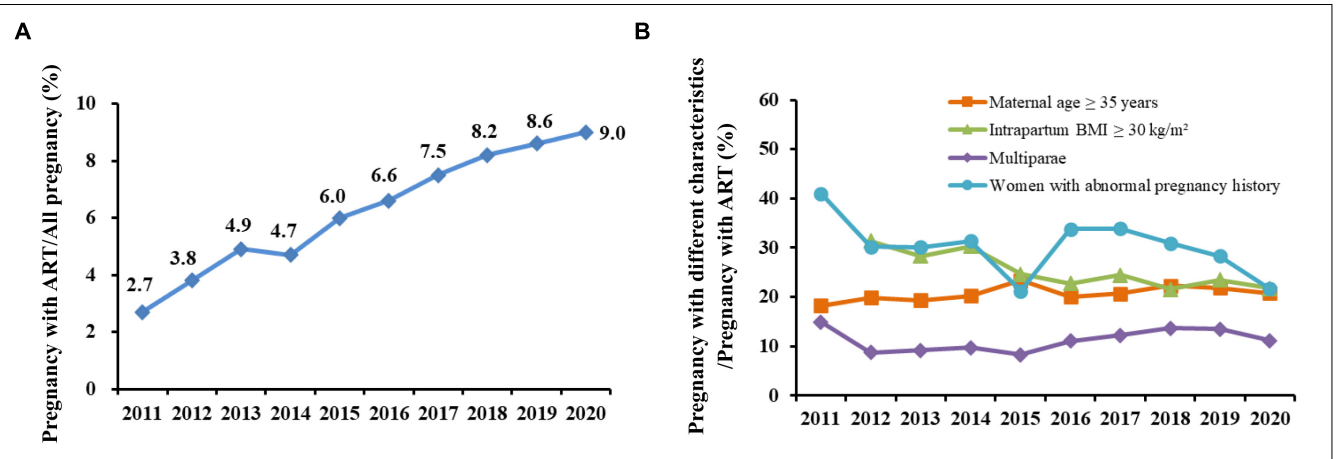

FIGURE 1 | Yearly trends in percentage of assisted reproductive technology (ART) pregnancy and pregnancy with different characteristics from 2011 to 2020. **(A)** Percentage of ART pregnancy among all pregnancy; **(B)** Percentage of different characteristics among ART pregnancy.

TABLE 1 | Maternal characteristics between naturally pregnancy and assisted reproductive technology (ART) pregnancy.

Maternal characteristics	All pregnancy (*n* = 211606)	Naturally pregnancy (*n* = 198002)	ART pregnancy (*n* = 13604)	Standardized difference
Maternal age [year, n (%)]	29.6 ± 3.9	29.5 ± 3.9	31.6 ± 3.9	0.547
<25	13274 (6.3)	13015 (6.6)	259 (1.9)	0.562
25-	104626 (49.4)	100616 (50.8)	4010 (29.5)	
30-	69047 (32.6)	62579 (31.6)	6468 (47.5)	
35-	21107 (10.0)	18711 (9.4)	2396 (17.6)	
40-	3550 (1.7)	3080 (1.6)	470 (3.5)	
Height (cm)	162.0 ± 4.7	162.1 ± 4.7	161.8 ± 4.7	0.066
Intrapartum weight (kg)	70.6 ± 9.1	70.5 ± 9.0	72.8 ± 9.9	0.252
Intrapartum BMI [kg/m^2, n (%)]	26.9 ± 3.2	26.8 ± 3.1	27.8 ± 3.5	0.309
<25	43730 (28.9)	41489 (29.5)	2241 (20.7)	0.287
25-	84527 (55.8)	78580 (55.9)	5947 (55)	
30-	20845 (13.8)	18569 (13.2)	2276 (21.1)	
35-	2323 (1.5)	1977 (1.4)	346 (3.2)	
Parity [n (%)]				0.345
Nulliparae	161328 (76.2)	149292 (75.4)	12036 (88.5)	
Multiparae	50278 (23.8)	48710 (24.6)	1568 (11.5)	
Birthplace [n (%)]				0.039
Jiangsu province	196035 (93.9)	183445 (93.9)	12590 (93.0)	
Other provinces	12773 (6.1)	11823 (6.1)	950 (7.0)	
Menstrual cycle [day, n (%)]				0.230
21-	181015 (91.6)	169954 (92.1)	11061 (84.8)	
36- or Irregularity	16496 (8.4)	14521 (7.9)	1975 (15.2)	
Abnormal pregnancy history [n (%)]	22559 (11.2)	18681 (9.9)	3878 (29.1)	0.500
Early abortion (≥2 times)	17181 (8.5)	14496 (7.7)	2685 (20.2)	0.366
Intermediate and late abortion or abnormal development	4205 (2.1)	3459 (1.8)	746 (5.6)	0.200
Ectopic pregnancy	3234 (1.6)	2114 (1.1)	1120 (8.4)	0.347
With uterine fibroids [n (%)]	11295 (5.3)	10160 (5.1)	1135 (8.3)	0.128

ART, assisted reproductive technology; BMI, body mass index.

TABLE 2 | The prevalence of pregnancy complications between naturally pregnancy and ART pregnancy.

Pregnancy complications [n (%)]	All pregnancy (*n* = 211606)	Naturally pregnancy (*n* = 198002)	ART pregnancy (*n* = 13604)	Standardized difference
Gestational diabetes	39498 (18.7)	35687 (18.0)	3811 (28.0)	0.239
Preeclampsia	5122 (2.4)	4382 (2.2)	740 (5.4)	0.169
Severe preeclampsia	2279 (1.1)	1943 (1.0)	336 (2.5)	0.114
Anemia	47080 (22.2)	43552 (22.0)	3528 (25.9)	0.092
Moderate or severe anemia	7583 (3.6)	6892 (3.5)	691 (5.1)	0.079
Liver-related diseases	8321 (3.9)	7605 (3.8)	716 (5.3)	0.068
Thyroid-related diseases	19323 (9.1)	17463 (8.8)	1860 (13.7)	0.154

Liver-related diseases included intrahepatic cholestasis, hepatitis, liver dysfunction, liver damage etc., thyroid-related diseases included hyperthyroidism, hypothyroidism, thyroiditis, thyroid tumor, etc. ART, assisted reproductive technology.

data were extracted and cleaned by using Natural Language Processing (NLP) technique (24). Maternal characteristics of all pregnancies were firstly extracted, including maternal age (year), height (cm), intrapartum weight (kg), parity, birthplace (Jiangsu province in China, other provinces in China or other countries), menstrual cycle (21–35 days, 36 days- or irregularity), abnormal pregnancy history and history of uterine fibroids. Maternal age was divided into five groups: <25, 25–29, 30–34, 35–39, ≥40 years. Intrapartum body mass index (BMI, kg/m^2) was calculated as maternal intrapartum weight divided by the square of height, and classified into four groups: <25, 25–29.9, 30–34.9, ≥35 kg/m^2. Parity did not include this pregnancy and was divided into 0 (nulliparae) and ≥1 (multiparae). Abnormal pregnancy history refers to a history of early abortion (≥2 times), intermediate and late abortion, abnormal development, or ectopic pregnancy.

We also used the HIS Database to obtain data on pregnancy complications, perinatal complications and neonatal outcomes. Data on pregnancy complications included gestational diabetes (fasting glucose concentrations ≥ 5.5 mmol/l or 2-h plasma glucose concentrations ≥ 8.0 mmol/l), preeclampsia (hypertension from 20 weeks' gestation and proteinuria; severe preeclampsia was defined as preeclampsia with either a diastolic blood pressure ≥ 110 mmHg or proteinuria ≥ 5 g/day or both), anemia (hemoglobin < 100 g/l and hematocrit < 0.30; moderate or severe anemia was defined as hemoglobin < 90 g/l or 60 g/l), liver-related diseases (cholestasis, hepatitis, liver function damage, etc.) and thyroid-related diseases (hyperthyroidism, hypothyroidism, thyroiditis, etc.). Data on perinatal complications included hospitalization time (day), preterm birth (<37 weeks' gestation), premature rupture of membrane, amniotic fluid pollution (clear as 0°, I°, II°, or III°), polyhydramnios (>2,000 ml in the third trimester), oligohydramnios (<300 ml in the third trimester), cord entanglement, torsion of cord, intrapartum fever (intrapartum temperature > 38°C), placenta previa, antepartum hemorrhage, postpartum hemorrhage (measured blood loss ≥ 500 ml) and delivery mode (spontaneous labor or cesarean section). And data on neonatal outcomes included gestational weeks in birth, offspring gender, birth weight (g), macrosomia (birth weight ≥ 4,000 g), twins or multiples, fetal distress, stillbirth or abnormal development (fetal malformation).

TABLE 3 | Perinatal complications between naturally pregnancy and ART pregnancy.

Perinatal complications	All pregnancy (*n* = 211606)	Naturally pregnancy (*n* = 198002)	ART pregnancy (*n* = 13604)	Standardized difference
Hospitalization time (day)	5.6 ± 2.3	5.6 ± 2.3	6.2 ± 2.6	0.274
Preterm birth [n (%)]	16054 (7.6)	13274 (6.7)	2780 (20.4)	0.409
Premature rupture of membrane [n (%)]	58537 (27.7)	55583 (28.1)	2954 (21.7)	0.147
Amniotic fluid pollution [n (%)]				0.178
Clear (0°)	134056 (83.0)	124330 (82.6)	9726 (88.6)	
I°	8630 (5.3)	8164 (5.4)	466 (4.2)	
II°	8872 (5.5)	8484 (5.6)	388 (3.5)	
III°	9944 (6.2)	9546 (6.3)	398 (3.6)	
Polyhydramnios [n (%)]	5697 (2.7)	5236 (2.6)	461 (3.4)	0.044
Oligohydramnios [n (%)]	13656 (6.5)	12693 (6.4)	963 (7.1)	0.027
Cord entanglement [n (%)]	76217 (36.0)	71562 (36.1)	4655 (34.2)	0.040
Torsion of cord [n (%)]	6522 (3.1)	6073 (3.1)	449 (3.3)	0.013
Intrapartum fever [n (%)]	23815 (11.3)	22529 (11.4)	1286 (9.5)	0.063
Placenta previa [n (%)]	11483 (5.4)	10328 (5.2)	1155 (8.5)	0.130
Antepartum hemorrhage [n (%)]	557 (0.3)	513 (0.3)	44 (0.3)	0.012
Postpartum hemorrhage [n (%)]	22586 (10.7)	19979 (10.1)	2607 (19.2)	0.259
Cesarean section [n (%)]	89560 (42.7)	79317 (40.5)	10243 (75.5)	0.759

ART, assisted reproductive technology.

Statistical Analyses

We compared maternal characteristics and pregnancy outcomes between group of ART pregnancy and group of naturally pregnancy. Continuous variables were described as mean and standard deviation ($\bar{x} \pm s$), and categorical variables were displayed as frequency (percentage). All comparisons between groups were conducted using standardized differences, which are not influenced by sample size and have been frequently used in previous large cohort studies (25–27). A standardized difference ≥ 0.1 indicates meaningful difference between groups. The association between ART using and pregnancy outcomes were evaluated by logistic regression analysis. The crude and adjusted odds ratio (OR) with 95% confidence intervals (95%CI) for pregnancy outcomes were calculated. Adjusted values were adjusted for maternal age, intrapartum BMI, parity, birth plurality and abnormal pregnancy history. All statistical analyses were two-sided and performed using R software (version 3.2.2).

TABLE 4 | Neonatal outcomes between naturally pregnancy and ART pregnancy.

Neonatal outcomes	All pregnancy (*n* = 211606)	Naturally pregnancy (*n* = 198002)	ART pregnancy (*n* = 13604)	Standardized difference
Gestational weeks	38.7 ± 1.9	38.8 ± 1.8	37.7 ± 2.4	0.519
Gender [n (%)]				
Boy	90392 (52.2)	83808 (52.2)	6584 (51.9)	0.098
Girl	82813 (47.8)	76713 (47.8)	6100 (48.1)	0.102
Birth weight (g)	3321.2 ± 507.9	3334.2 ± 495.2	3139.6 ± 632.2	0.343
Macrosomia [n (%)]	15301 (7.2)	14496 (7.3)	805 (5.9)	0.056
Twins or multiples [n (%)]	5201 (2.5)	2387 (1.2)	2814 (20.7)	0.657
Fetal distress [n (%)]	12956 (6.8)	12246 (6.9)	710 (5.5)	0.059
Stillbirth or abnormal development [n (%)]	2299 (1.1)	1845 (0.9)	454 (3.3)	0.167

ART, assisted reproductive technology.

RESULTS

A total of 211,606 women were included in this retrospective analysis, of whom 13,604 women conceived by ART as group of ART pregnancy, and 198,002 women conceived naturally without ART as group of naturally pregnancy. Over the past 10 years, the proportion of ART pregnancy has increased each year, reaching a peak in 2020 (9.0%) (**Figure 1A**). Of the ART pregnancy, the proportion of pregnant women over 35 years old and multiparae increased mildly, while the proportion of women with intrapartum BMI greater than 30 kg/m^2 decreased slightly. And with the implementation of the universal two-child policy in 2015, the proportion of women with abnormal pregnancy history in ART pregnancy decreased sharply, and then increased rapidly (**Figure 1B**).

Maternal characteristics between ART and naturally pregnancy is summarized in **Table 1**. The mean maternal age and intrapartum BMI of women conceived by ART were significantly higher than those of women conceived naturally (standardized difference = 0.547 and 0.309, respectively). And women conceived by ART were more likely to be nulliparae (88.5% *vs.* 75.4%, standardized difference = 0.345), more likely to have a long or irregular menstrual cycle (15.2% *vs.* 7.9%, standardized difference = 0.230) and an abnormal pregnancy history (including early abortion, intermediate and late abortion, abnormal development, or ectopic pregnancy, 29.1% *vs.* 9.9%, standardized difference = 0.500), and more likely to have uterine fibroids (8.3% *vs.* 5.1%, standardized difference = 0.128). There were no significant standardized differences in maternal height and birthplace between the two groups.

TABLE 5 | The association of ART with maternal and offspring health.

Maternal and offspring health	Naturally pregnancy	Univariate		Multivariate	
		ART pregnancy	*P*	ART pregnancy	*P*
Pregnancy complications					
Gestational diabetes (*n* = 39498)	1.0 (ref)	1.77 (1.70–1.84)	<0.001	1.39 (1.33–1.46)	<0.001
Preeclampsia (*n* = 5122)	1.0 (ref)	2.54 (2.35–2.75)	<0.001	1.26 (1.14–1.41)	<0.001
Severe preeclampsia (*n* = 2279)	1.0 (ref)	2.56 (2.27–2.87)	<0.001	1.12 (0.96–1.31)	0.146
Anemia (*n* = 47080)	1.0 (ref)	1.24 (1.19–1.29)	<0.001	1.01 (0.96–1.06)	0.678
Moderate or severe anemia (*n* = 7583)	1.0 (ref)	1.48 (1.37–1.61)	<0.001	1.20 (1.08–1.32)	<0.001
Liver-related diseases (*n* = 8321)	1.0 (ref)	1.39 (1.29–1.50)	<0.001	1.14 (1.03–1.26)	0.012
Thyroid-related diseases (*n* = 19323)	1.0 (ref)	1.64 (1.56–1.72)	<0.001	1.29 (1.21–1.37)	<0.001
Perinatal complications					
Preterm birth (*n* = 16054)	1.0 (ref)	3.58 (3.42–3.74)	<0.001	1.61 (1.49–1.74)	<0.001
Premature rupture of membrane (*n* = 58537)	1.0 (ref)	0.71 (0.68–0.74)	<0.001	0.66 (0.62–0.71)	<0.001
Amniotic fluid pollution					
I° (*n* = 8630)	1.0 (ref)	0.73 (0.66–0.80)	<0.001	0.83 (0.74–0.92)	0.001
II° (*n* = 8872)	1.0 (ref)	0.58 (0.53–0.65)	<0.001	0.66 (0.59–0.74)	<0.001
III° (*n* = 9944)	1.0 (ref)	0.53 (0.48–0.59)	<0.001	0.57 (0.51–0.64)	<0.001
Polyhydramnios (*n* = 5697)	1.0 (ref)	1.29 (1.17–1.42)	<0.001	1.00 (0.89–1.13)	0.992
Oligohydramnios (*n* = 13656)	1.0 (ref)	1.11 (1.04–1.19)	0.002	1.00 (0.92–1.08)	0.968
Cord entanglement (*n* = 76217)	1.0 (ref)	0.92 (0.89–0.95)	<0.001	0.89 (0.85–0.93)	<0.001
Torsion of cord (*n* = 6522)	1.0 (ref)	1.08 (0.98–1.19)	0.128	0.93 (0.83–1.05)	0.235
Intrapartum fever (*n* = 23815)	1.0 (ref)	0.81 (0.77–0.86)	<0.001	0.69 (0.64–0.74)	<0.001
Placenta previa (*n* = 11483)	1.0 (ref)	1.69 (1.58–1.80)	<0.001	1.48 (1.37–1.60)	<0.001
Antepartum hemorrhage (*n* = 557)	1.0 (ref)	1.25 (0.92–1.70)	0.157	0.82 (0.50–1.34)	0.421
Postpartum hemorrhage (*n* = 22586)	1.0 (ref)	2.11 (2.02–2.21)	<0.001	1.14 (1.08–1.21)	<0.001
Cesarean section (*n* = 79317)	1.0 (ref)	4.53 (4.35–4.72)	<0.001	2.84 (2.70–2.99)	<0.001
Neonatal outcomes					
Macrosomia (*n* = 15301)	1.0 (ref)	0.80 (0.74–0.86)	<0.001	0.88 (0.80–0.95)	0.002
Twins or multiples (*n* = 5201)	1.0 (ref)	21.37 (20.17–22.65)	<0.001	24.20 (22.43–26.11)	<0.001
Fetal distress (*n* = 12956)	1.0 (ref)	0.78 (0.72–0.84)	<0.001	0.66 (0.60–0.73)	<0.001
Stillbirth or abnormal development (*n* = 2299)	1.0 (ref)	3.67 (3.31–4.07)	<0.001	2.76 (2.39–3.17)	<0.001

All values are ORs (95% CIs). Values were determined by using logistic regression. Adjusted values were adjusted for maternal age, intrapartum BMI, parity, birth plurality and abnormal pregnancy history. For odds of twins or multiples, adjusted values were adjusted for maternal age, intrapartum BMI, parity and abnormal pregnancy history.

The incidences of pregnancy and perinatal complications in ART and naturally pregnancy was exhibited in **Tables 2**, **3**. Statistically significant increases were noted in gestational diabetes (28.0%), preeclampsia (5.4%), thyroid-related diseases (13.7%), preterm birth (20.4%), placenta previa (8.5%), postpartum hemorrhage (19.2%) and cesarean section (75.5%) in ART pregnancy, compared to naturally pregnancy (standardized difference > 0.1). The occurring rates of anemia (25.9%), liver-related diseases (5.3%), polyhydramnios (3.4%), oligohydramnios (7.1%) and torsion of cord (3.3%) were also elevated in ART pregnancy, but with no significant difference (standardized difference < 0.1). In contrast, there was a decline in the incidences of premature rupture of membrane (21.7%) and amniotic fluid pollution (I°: 4.2%, II°: 3.5%, III°: 3.6%) in ART pregnancy (standardized difference > 0.1). We also analyzed neonatal outcomes between naturally pregnancy and ART pregnancy (**Table 4**). The mean birth weight of ART pregnancy was significantly lower than that of naturally pregnancy (standardized difference = 0.343). Moreover, significant rises of incidence were observed in twins or multiples (20.7%) and stillbirth or abnormal development (3.3%) in ART pregnancy (standardized difference > 0.1). No significant difference was noted in macrosomia and fetal distress between the two groups.

Multivariable logistic regression analysis showed that the association between ART and pregnancy outcomes were significant (**Table 5**). Nearly all the pregnancy complication listed, including gestational diabetes [aOR (95%CI) = 1.39 (1.33–1.46)], preeclampsia [aOR (95%CI) = 1.26 (1.14–1.41)], moderate or severe anemia [aOR (95%CI) = 1.20 (1.08–1.32)], liver-related diseases [aOR (95%CI) = 1.14 (1.03–1.26)], and thyroid-related diseases [aOR (95%CI) = 1.29 (1.21–1.37)], were more likely to occur among women conceived by ART. In terms of perinatal complications, the risk of preterm birth [aOR (95%CI) = 1.61 (1.49–1.74)], placenta previa [aOR (95%CI) = 1.48 (1.37–1.60)], postpartum hemorrhage [aOR (95%CI) = 1.14 (1.08–1.21)], and cesarean section [aOR (95%CI) = 2.84 (2.70–2.99)] were significantly increased, while the risk of premature rupture of membrane [aOR (95%CI) = 0.66 (0.62–0.71)], amniotic fluid pollution [I°: aOR (95%CI) = 0.83 (0.74–0.92); II°: aOR

TABLE 6 | Stratified analysis on the association of ART with maternal and offspring health by birth plurality.

Maternal and offspring health	Naturally pregnancy	ART pregnancy		
		Singletons	Twins or multiples	*P*
Pregnancy complications				
Gestational diabetes	1.0 (ref)	1.40 (1.33–1.47)	1.34 (1.12–1.61)	0.648
Preeclampsia	1.0 (ref)	1.27 (1.12–1.43)	1.24 (0.99–1.56)	0.856
Severe preeclampsia	1.0 (ref)	1.11 (0.92–1.34)	1.10 (0.81–1.48)	0.960
Anemia	1.0 (ref)	1.00 (0.94–1.05)	1.15 (0.99–1.33)	0.082
Moderate or severe anemia	1.0 (ref)	1.17 (1.05–1.31)	1.35 (1.02–1.79)	0.353
Liver-related diseases	1.0 (ref)	1.17 (1.04–1.30)	1.06 (0.82–1.37)	0.489
Thyroid-related diseases	1.0 (ref)	1.30 (1.22–1.39)	1.21 (0.98–1.50)	0.528
Perinatal complications				
Preterm birth	1.0 (ref)	1.64 (1.49–1.80)	1.44 (1.24–1.66)	0.142
Premature rupture of membrane	1.0 (ref)	0.65 (0.61–0.70)	0.91 (0.71–1.16)	0.010
Amniotic fluid pollution				
I°	1.0 (ref)	0.83 (0.74–0.93)	0.81 (0.45–1.47)	0.937
II°	1.0 (ref)	0.67 (0.60–0.76)	0.40 (0.18–0.90)	0.214
III°	1.0 (ref)	0.58 (0.52–0.65)	0.29 (0.13–0.67)	0.101
Polyhydramnios	1.0 (ref)	1.09 (0.96–1.24)	0.72 (0.53–0.97)	0.013
Oligohydramnios	1.0 (ref)	1.02 (0.94–1.10)	0.96 (0.68–1.36)	0.738
Cord entanglement	1.0 (ref)	0.88 (0.84–0.92)	1.08 (0.93–1.26)	0.011
Torsion of cord	1.0 (ref)	0.96 (0.85–1.09)	0.68 (0.45–1.03)	0.118
Intrapartum fever	1.0 (ref)	0.68 (0.63–0.73)	1.09 (0.82–1.47)	0.002
Placenta previa	1.0 (ref)	1.46 (1.35–1.58)	1.96 (1.28–3.00)	0.183
Antepartum hemorrhage	1.0 (ref)	0.63 (0.35–1.15)	3.86 (0.73–20.25)	0.044
Postpartum hemorrhage	1.0 (ref)	1.17 (1.10–1.25)	1.04 (0.90–1.21)	0.152
Cesarean section	1.0 (ref)	2.92 (2.77–3.08)	1.23 (0.93–1.63)	<0.001
Neonatal outcomes				
Fetal distress	1.0 (ref)	0.69 (0.62–0.76)	0.55 (0.39–0.78)	0.219
Stillbirth or abnormal development	1.0 (ref)	3.48 (3.01–4.03)	0.82 (0.60–1.14)	<0.001

All values are ORs (95% CIs). Values were determined by using logistic regression. Adjusted values were adjusted for maternal age, intrapartum BMI, parity and abnormal pregnancy history. The P-values were calculated for heterogeneity test.

TABLE 7 | Stratified analysis on the association of ART with maternal and offspring health by maternal age.

Maternal and offspring health	Naturally pregnancy	ART pregnancy		
		Maternal age < 35 y	Maternal age ≥ 35 y	*P*
Pregnancy complications				
Gestational diabetes	1.0 (ref)	1.59 (1.50 – 1.68)	1.45 (1.31 – 1.60)	0.116
Preeclampsia	1.0 (ref)	1.34 (1.19 – 1.52)	1.19 (0.94 – 1.50)	0.378
Severe preeclampsia	1.0 (ref)	1.15 (0.96 – 1.37)	1.14 (0.82 – 1.58)	0.963
Anemia	1.0 (ref)	1.02 (0.97 – 1.08)	1.02 (0.91 – 1.15)	1.000
Moderate or severe anemia	1.0 (ref)	1.27 (1.13 – 1.41)	1.24 (0.97 – 1.58)	0.861
Liver-related diseases	1.0 (ref)	1.15 (1.02 – 1.29)	1.18 (0.95 – 1.47)	0.839
Thyroid-related diseases	1.0 (ref)	1.32 (1.23 – 1.42)	1.39 (1.21 – 1.60)	0.519
Perinatal complications				
Preterm birth	1.0 (ref)	1.56 (1.43 – 1.70)	1.26 (1.06 – 1.51)	0.034
Premature rupture of membrane	1.0 (ref)	0.70 (0.65 – 0.75)	0.67 (0.57 – 0.80)	0.641
Amniotic fluid pollution				
I°	1.0 (ref)	0.81 (0.72 – 0.91)	0.96 (0.74 – 1.24)	0.240
II°	1.0 (ref)	0.68 (0.60 – 0.78)	0.71 (0.53 – 0.94)	0.788
III°	1.0 (ref)	0.62 (0.54 – 0.70)	0.53 (0.38 – 0.74)	0.390
Polyhydramnios	1.0 (ref)	0.94 (0.82 – 1.07)	1.40 (1.11 – 1.77)	0.004
Oligohydramnios	1.0 (ref)	1.03 (0.94 – 1.13)	0.93 (0.76 – 1.12)	0.351
Cord entanglement	1.0 (ref)	0.91 (0.87 – 0.96)	0.86 (0.78 – 0.96)	0.335
Torsion of cord	1.0 (ref)	0.98 (0.86 – 1.11)	1.04 (0.78 – 1.37)	0.706
Intrapartum fever	1.0 (ref)	0.74 (0.68 – 0.80)	0.78 (0.66 – 0.93)	0.587
Placenta previa	1.0 (ref)	1.77 (1.61 – 1.94)	1.24 (1.06 – 1.44)	<0.001
Antepartum hemorrhage	1.0 (ref)	0.89 (0.51 – 1.56)	0.80 (0.29 – 2.25)	0.858
Postpartum hemorrhage	1.0 (ref)	1.27 (1.19 – 1.35)	1.07 (0.94 – 1.22)	0.020
Cesarean section	1.0 (ref)	3.17 (3.00 – 3.35)	2.39 (2.08 – 2.76)	<0.001
Neonatal outcomes				
Fetal distress	1.0 (ref)	0.75 (0.68 – 0.83)	0.58 (0.45 – 0.75)	0.066
Stillbirth or abnormal development	1.0 (ref)	2.48 (2.13 – 2.88)	2.25 (1.52 – 3.32)	0.649

All values are ORs (95% CIs). Values were determined by using logistic regression. Adjusted values were adjusted for intrapartum BMI, parity, birth plurality and abnormal pregnancy history. The P-values were calculated for heterogeneity test.

(95%CI) = 0.66 (0.59–0.74); III°: aOR (95%CI) = 0.57 (0.51–0.64)], cord entanglement [aOR (95%CI) = 0.89 (0.85–0.93)], and intrapartum fever [aOR (95%CI) = 0.69 (0.64–0.74)] were significantly decreased in ART pregnancy as compared with naturally pregnancy. For neonatal outcomes, women conceived by ART were more likely to have twins or multiples [aOR (95%CI) = 24.20 (22.43–26.11)], and the risk of stillbirth or abnormal development [aOR (95%CI) = 2.76 (2.39–3.17)] was also significantly increased. Moreover, the risk of macrosomia [aOR (95%CI) = 0.88 (0.80–0.95)] and fetal distress [aOR (95%CI) = 0.66 (0.60–0.73)] were significantly decreased in ART pregnancy (**Table 5**).

The association of ART with maternal and neonatal outcomes were also evaluated by stratifying on birth plurality and maternal age (**Tables 6**, **7**). When restriction to singletons, the risks of adverse pregnancy outcomes as listed above were reduced. And the effects of ART on the risk of premature rupture of membrane, cord entanglement, intrapartum fever, cesarean section, and stillbirth or abnormal development (ART pregnancy vs. naturally pregnancy) were more pronounced among singleton pregnancies compared with that among pregnancies of twins or multiples, while the effect of ART on the risk of polyhydramnios was more prominent among pregnancies of twins or multiples (heterogeneity test: $P < 0.05$). When stratified by maternal age, we found the effects of ART on the risk of preterm birth, placenta previa, postpartum hemorrhage and cesarean section (ART pregnancy vs. naturally pregnancy) were more pronounced among women under 35 years compared with that among women over 30 years, while the effect of ART on the risk of polyhydramnios was more prominent among women over 35 years (heterogeneity test: $P < 0.05$).

DISCUSSION

This retrospective, hospital-based cohort study including 13,604 ART pregnancies and 198,002 naturally pregnancies was conducted in Nanjing, China from 2011 to 2020. The study showed the widespread application of ART in China, with the proportion of ART pregnancies increasing year by year in the past decade, and confirmed the increased risks of several adverse pregnancy outcomes in ART pregnancies. We found a 24.2-fold increase in the incidence of multiple births in ART pregnancies compared to naturally pregnancies, then stratified the analysis by birth plurality, suggesting multiple births are indeed an important factor leading to adverse pregnancy outcomes.

In the present study, the increased risks were found in ART pregnancy compared with naturally pregnancy: gestational diabetes (1.39-fold), preeclampsia (1.26-fold), moderate or severe anemia (1.20-fold), liver-related diseases (1.14-fold), thyroid-related diseases (1.29-fold), preterm birth (1.61-fold), placenta previa (1.48-fold), postpartum hemorrhage (1.14-fold), cesarean section (2.84-fold), and stillbirth or abnormal development (2.76-fold), which were largely consistent with the findings of previous studies (10, 28–32). Although these risks were reduced when restriction to singletons, significant differences remained. Some studies have suggested that infertility is one of the risk factors for adverse pregnancy outcomes (33). However, infertility factors cannot fully explain the associations. For infertile women, women conceived with ART had an increased risks of adverse pregnancy outcomes compared with women conceived with non-ART (34). Therefore, some researchers believe that the increased risks of adverse outcomes after ART conception are mainly related to ART manipulation factors (6), which is due to the addition of many non-physiological operations by ART. For example, the type of ovulation induction drugs used in the early stage, the composition of the culture medium, the storage time in the culture medium, the freezing and dissolution process of the embryo, polyspermic fertilization, and the hormone level at the time of implantation, all play an important role in the occurrence of adverse pregnancy outcomes (35). Other studies have pointed out that different methods of ART may lead to different types of adverse pregnancy outcomes (36). At the same time, the longer and more times of ART treatment, the greater the harm to women and their offspring (36). In addition, ART pregnancies may be more closely monitored than naturally pregnancies, which partly explains the higher incidence of adverse pregnancy outcomes in ART pregnancies (37). However, current studies and evidence cannot fully elucidate the mechanism by which ART increases the risk of adverse pregnancy outcomes, and the specific mechanism needs further research.

The main advantage of this study was the large sample size of pregnancy, which allowed us to conduct further subgroup analysis with enough power. And the data obtained from HIS database by using NLP technique is of high quality. However, there are some limitations in this study. First, the population we studied was limited to one city in eastern China (Nanjing). Therefore, we should be cautious in generalizing our findings to other regions. Second, we did not collect information on the ART form. The more intricate and invasive the ART form used, the more likely it was to cause adverse pregnancy outcomes. And the records of pre-pregnancy BMI, baseline endocrine level, causes of infertility, ovarian stimulation protocols, and quality of transferred embryos lacked in our database, were not included in this study. Third, the retrospective design of this study could not assess a causal relationship between ART and adverse pregnancy outcomes. These limitations should be considered in future studies.

CONCLUSION AND PROSPECT

Women conceived by ART were at increased risks of several adverse pregnancy outcomes compared with women conceived naturally. Multiple pregnancies due to multiple embryos transferred could partly explain the increased risks. The transfer of single embryo of high quality should be promoted. However, ART singleton pregnancy still showed higher risks of several adverse pregnancy outcomes compared with naturally pregnancy, suggesting ART itself is also significantly correlated with pathological pregnancy. Therefore, policies related to ART indications should be strictly formulated to reverse the high rate of ART pregnancy. Given our findings, prenatal and intrapartum monitoring should be strengthened, and neonatal outcomes should be closely observed for ART pregnancy. And more research should be conducted to further clarify whether the

increased risk of adverse pregnancy outcomes is due to ART itself, multiple births, or potential infertility.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board of Women's Hospital of Nanjing Medical University (2020KY-011). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JW initiated, conceived, and supervised the study. WT and LH did data collection and performed the data analysis. All authors approved the final format of the submitted manuscript.

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Parent Joint AB Blood Group Is Associated With Clinical Outcomes of *in vitro* Fertilization and Intracytoplasmic Sperm Injection Treatment in Chinese Women

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Background: A number of publications have examined the relation between blood group and female infertility including ovarian reserve, recurrent miscarriage, and live birth. However, there is a lack of literature investigating joint mother/father ABO blood type in a large cohort. This study aimed to investigate the association between couple combinations for ABO blood groups and assisted reproductive technology (ART) outcomes in patients undergoing *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI).

Methods: This retrospective cohort study included 30,717 couples who underwent IVF cycles between 2010 and 2019. The clinical outcomes of IVF treatment were the primary outcome. History of spontaneous miscarriage, embryo quality, and birth sex, weights, defects rate were also studied.

Results: There was no difference in the baseline demographics between the blood type groups. There was a statistically significant positive association between the combination of female blood type AB and male blood type AB with biochemical pregnancy, clinical pregnancy, and live birth rate (OR 1.36; 95% CI, 1.05–1.78; $P = 0.02$ and OR 1.31; 95% CI, 1.0–1.68; $P = 0.031$ and OR 1.28; 95% CI, 1.01–1.63; $P = 0.041$ respectively). No statistically significant difference was observed between joint mother/father ABO blood types and high-quality embryo rate, early abortion rate, birth sex, birth weights, and birth defect rate.

Conclusions: Our findings suggest that the success rate of IVF/ICSI cycles in parent mating AB blood type is higher than that in other blood type combination groups.

Keywords: clinical outcomes, *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), husband/wife joint ABO blood type, success rate

INTRODUCTION

ABO blood group antigen is a complex molecule expressed on the surface of human red blood cells and some other cell types and various tissues. They play a major role in transfusion medicine, meanwhile, increasing evidence suggests that the ABO blood group is related to the development of many human diseases. A number of publications have studied the relation between blood group and female infertility including ovarian reserve, recurrent miscarriage, and live birth.

As early as 1960, some authors proposed that ABO blood incompatibility may be associated with infertility (1). In 1967 Schwimmer et al. suggested that ABO blood group incompatibility was one of the possible immunologic causes of infertility, they found that compared with couples with organic causes of infertility, couples with primary and secondary unexplained infertility possessed a higher incidence of ABO incompatibility. However, the association between ABO blood groups and infertility has been a point of controversy, while some studies support the absence of any relationship between blood groups and infertility in different populations. The correlation of blood groups to ovarian reserve has been investigated by multiple studies. Nejat et al. showed that blood type O was associated with a diminished ovarian reserve and that the A blood group antigen appears to be protective of ovarian reserve (2). On the contrary, Lin et al. (3) showed that Chinese women with blood type O had a less diminished ovarian reserve than women with blood types B and AB, who had more diminished ovarian reserve while blood type A was not associated with ovarian reserve. Other studies could not confirm this and show no correlation of blood groups to ovarian reserve response during *in vitro* fertilization (IVF) treatment (4–7). Recently, a systematic review and meta-analysis was conducted, which suggested that ABO blood groups are not associated with ovarian reserve, Ovarian hyperstimulation syndrome (OHSS), and outcomes of assisted reproductive technology (ART) (8).

Two retrospective studies on infertile women undergoing IVF indicated conflicting conclusions about whether live birth relates to ABO blood type (9, 10). Some studies suggest that ABO blood type has an impact on female infertility, which is related to the incidence of ovarian hyperstimulation syndrome and endometriosis, important influence factors of pregnancy. A predominance of blood group A was shown in patients with endometriosis by studying 231 women with endometriosis and 166 infertile women without endometriosis (11). One case-control study on 121 Caucasian patients showed a positive association between A blood group and early-onset ovarian hyperstimulation syndrome (12).

Despite the aforementioned findings, there is a lack of literature investigating joint parent ABO blood types in a large cohort. This study explores whether ABO blood type is associated with the clinical outcomes of IVF treatment, history of spontaneous miscarriage, embryo quality parameters, and birth sex, weight, and birth defect rates in Chinese infertile couples.

MATERIALS AND METHODS

Study Population

This retrospective cohort study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University. Patients undergoing cycles of IVF/intracytoplasmic sperm injection (ICSI) at the Reproductive Medical Center, First Affiliated Hospital of Zhengzhou University, between January 2010 and December 2019 were included in this study. Follow-up data was finished in December 2020 and data took place in March 2021. From the initial pool of 39,477 cycles, 39,154 cycles have complete ABO and Rh blood type information with female patients aged >25 years and <35 years. Potential confounders may cause spontaneous abortion or affect ovarian response and ART outcomes were excluded in this study: chromosome abnormality ($n = 976$), cervical incompetence ($n = 345$), uterine malformation ($n = 1{,}031$), history of endometriosis or ovarian surgery ($n = 581$), atypical hyperplasia of endometrium ($n = 2$), hydatidiform mole ($n = 15$), and cervical cancer ($n = 2$), congenital disease ($n = 258$), amenorrhea ($n = 25$), POI/oocyte maturation disorder (36), submucous myoma ($n = 216$). Males with diagnoses of azoospermia ($n = 1{,}981$) and necrozoospermia ($n = 16$) were excluded. The blood type of donors of semen in artificial insemination (AID) or IVF with frozen semen donor (IVF-D) cannot be tracked. Males with severe oligoasthenospermia ($n = 2{,}953$) were also excluded to eliminate the influence of sperm quality on fertilization and ART outcomes. A total of 30,717 cycles met the inclusion criteria. Information on patients' characteristics, ART outcomes, and birth information for each couple were collected from the Clinical Reproductive Medicine Management System/Electronic Medical Record Cohort Database of the Reproductive Medical Center of the First Affiliated Hospital of Zhengzhou University (**Figure 1**).

Clinical and Laboratory Protocols

A blood type and screen were obtained in all patients as part of the initial infertility workup at the hospital's laboratory. Controlled ovarian hyperstimulation (COH), human chorionic gonadotropin (hCG) trigger, oocyte retrieval, embryo culture, and embryo transfer (ET) performed based on established protocols (13).

The use of GnRH-a differed in the two COH protocols: for GnRH-a prolonged protocol, 3.75 mg of GnRH-a was injected on day 2 of the menstrual cycle. For the short GnRH-a long protocol, 0.1 mg of GnRH-a was injected daily from the mid-luteal phase. When the patient achieved the criteria for pituitary suppression, Gonadotropin (Gonal-F, Merck, Germany; Puregon, Organon, Netherlands; Urofollitropin, Livzon, China) was used to initiate ovarian stimulation with the dose range of 75–300 IU based on the ovarian response (75–150 IU for normal or high ovarian response; 150–300 IU for reduced ovarian response). The exact dose of gonadotropin was adjusted based on age, body mass index (BMI, kg/m^2), antral follicle count (AFC), and anti-Müllerian hormone (AMH) level of the individual patient. hCG (Livzon) was injected with the oocyte maturation trigger and was administered when at least two follicles had reached 18 mm.

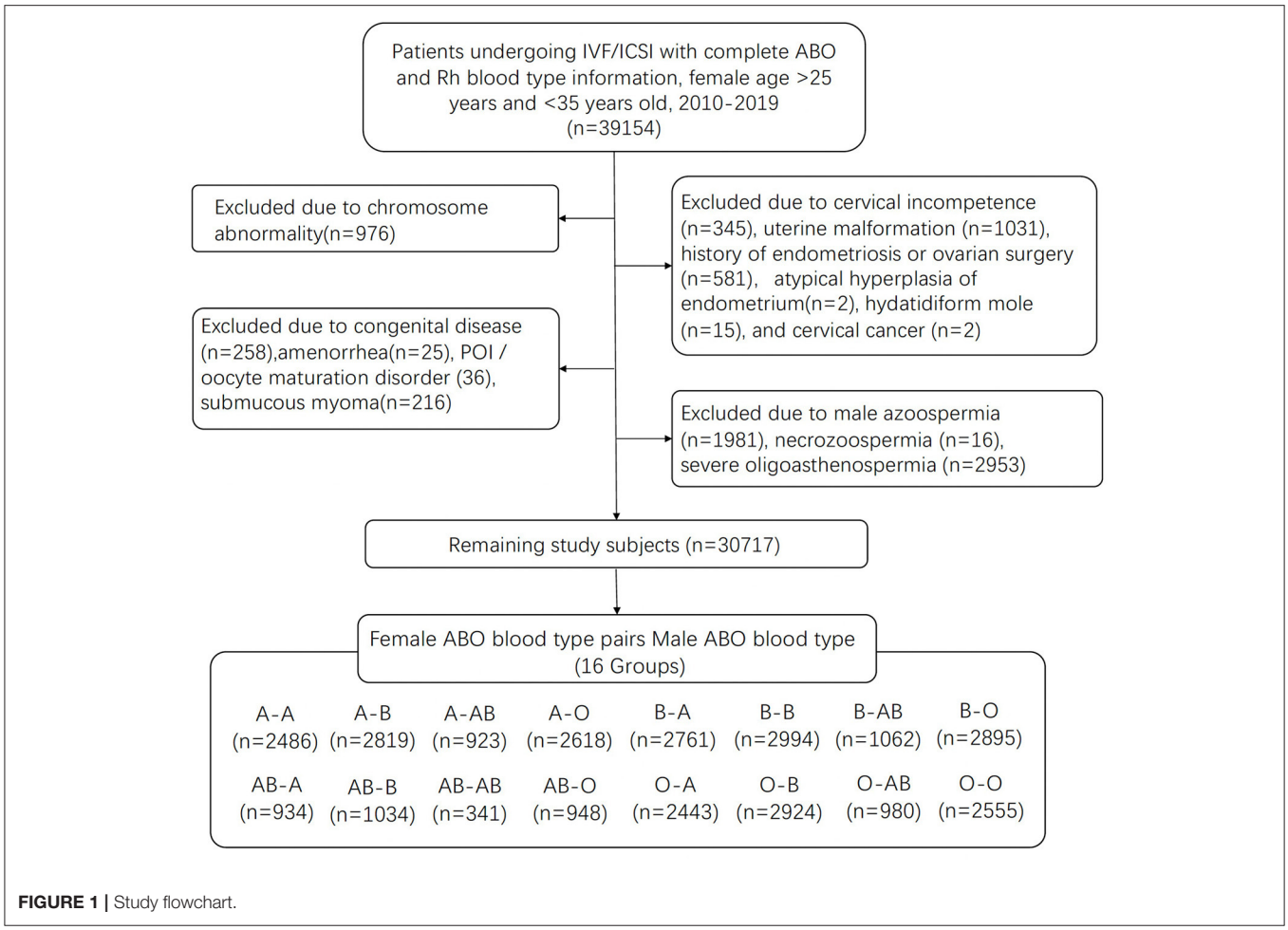

FIGURE 1 | Study flowchart.

Oocytes were collected by a transvaginal ultrasound-guided puncture at 36–37 h after hCG injection. Progesterone in oil was used for luteal support at a dose of 60 mg per day after oocyte pick-up.

Based on the condition of semen and the couples' reproductive history, fertilization was carried out with short-time insemination or ICSI insemination (14). The embryo morphology in the cleavage stage was observed and graded according to Peter's standards (15) on day 3. Good quality embryos were defined when they graded better than II. Day 5 blastocyst-stage embryos were graded according to the Gardner standard (16). The condition and the willingness of the patients were comprehensively considered to decide whether to perform fresh ET or frozen-thawed embryo transfer (FET).

Based on the regularity of the menstrual cycle, FET cycles were divided into natural cycles and artificial cycles. For natural cycles, patients were allocated to undergo ultrasonic evaluation starting from day 8–9 of the menstrual cycle. When the diameter of the dominant follicle was 16–20 mm, a blood sample test for progesterone (P) and luteinizing hormone (LH) levels was conducted to monitor ovulation. Thawing and transferring were performed 3 days for cleavage-stage embryos or 5 days for blastocyst-stage embryos after ovulation. Intramuscular (im) progesterone (40 mg) starting on the day of ovulation and oral dydrogesterone (20 mg) starting on the embryo transfer day were used for luteal support. For artificial cycles, patients began oral estradiol [2 mg, Progynova; Bayer, Leverkusen, Germany] twice a day on cycle day 3. This dose was adjusted based on the endometrial thickness every 4 days. After 12–14 days, an ultrasound was performed and a serum progesterone level was determined. If no leading follicle was present, progesterone (60 mg, im) and oral dydrogesterone [10 mg [this dose was changed to 20 mg 2 days later]] would be added to the regimen.

The serum hCG biochemical pregnancy tests were performed 14 and 18 days after the embryo transfer. For patients with hCG was more than 50 IU/L, transvaginal ultrasound was performed 35 days after embryo transfer.

Study Variables

Baseline demographics recorded for each patient included age (years), BMI (kg/m^2), infertility diagnosis, ABO blood type, and rhesus factor type. The primary outcome of this study was the clinical outcomes of IVF treatment. Biochemical pregnancy was defined as an increase of HCG levels higher than a detailed range at 14 days after embryo transfer. Clinical pregnancy was defined as one or multiple gestational sac(s) and cardiac activity seen via ultrasound 35 days after embryo transfer. Live birth was defined

as any birth event in which at least one baby is born alive. Early spontaneous miscarriage was defined as a pregnancy loss before 14 weeks of gestation following clinical pregnancy. Recurrent spontaneous abortion (RSA) was defined as the occurrence of at least two spontaneous losses before fetal viability.

Statistical Analysis

Association analyses were performed using custom R scripts. Results for continuous data are given as mean ± standard deviation (SD). Results for categorical variables are given as the number of cases (*n*) with a percentage of occurrence (%). The proportions of the different blood types were compared by the chi-squared test. Analysis of variance (ANOVA) was used for parametric data between blood type groups. Previous spontaneous abortion, embryo quality parameters, ART outcomes, birth sex, weight (log2 transformed), and defects were compared by blood type groups using Multivariable logistic regression models while adjusting for potential confounders. The study population was divided into 16 groups with female ABO blood type pairs and male ABO blood types. The association between one specific blood type group and clinical outcomes were analyzed when all the other blood type group were used as controls. ORs with 95% CIs were estimated from the model. Three models were used to test spontaneous abortion association: (1) a dosage model, which was treated as recurrent miscarriage, marked 1 and as O; (2) a dominant model, which was treated 0 as absent and more than 1 abortion as present; and (3) a continuous model to study the number of spontaneous abortions. $P < 0.05$ was considered to be statistically significant. The Bonferroni correction was used for the *P*-value correction of multiple comparisons.

RESULTS

The distribution of the study cohort based on blood type was as follows: 8,846 (28.80%) females with blood type A; 3,257 (10.60%) females with blood type AB; 9,712 (31.62%) females with blood type B; and 8,902(28.98%) females with blood type O. For Rh blood type, 98 (0.032%) females with blood type Rh-; 30,619 (99.68%) females with blood type Rh+; This distribution of blood types were similar to the male participant results: 8,624 (28.08%) male participants with blood type A; 3,257 (10.76%) male participants with blood type AB; 9,771 (31.81%) male participants with blood type B; and 9,016 (29.35%) male participants with blood type O. For Rh blood type, 120 (0.039%) female participants with blood type Rh−; 330,597 (99.61%), female participants with blood type Rh+. As shown in **Supplementary Table 1**, there was no difference in the distribution of blood type between infertile female and male participants.

Demographic and clinical characteristics of the study population are shown in **Table 1** and **Supplementary Table 1**. The women's average age was 30.0 ± 2.83 years. There was no difference in the age, BMI, duration of subfertility, primary subfertility, infertility diagnoses, basal sex hormone, COH and FET Protocols, peak estradiol level on the day of hCG trigger, peak endometrial thickness, number of retrieved oocytes, percentage of MII oocytes, good-quality embryos, number of embryos transferred, stage of embryos transferred by blood type in female groups. *P*-value of recombinant follicle stimulating hormone (rFSH) dosage ($P = 0.049$) and the percentages of fresh embryo transfer or frozen-thawed embryo transfer ($P = 0.046$) were <0.05, but there was no statistical difference when the two groups were compared.

Given the small sample size of patients with negative Rh in the population, especially when it was split into the four ABO blood groups, the rhesus factor was not included in the downstream association test.

History of Spontaneous Miscarriage Association Analysis

Potential interaction between the ABO blood group of mother/father mating and the history of spontaneous miscarriage were investigated by three models as described before. There was a statistically significantly higher percentage of the combination of female blood type AB and male blood type O in couples with at least one spontaneous miscarriage history (odds ratio [OR] 1.03; 95% confidence interval [CI], 1–1.05; $P = 0.0234$) than for the rest. However, no statistically significant difference was observed in Dosage and Continuous models (OR 1.01; 95% CI, 1–1.01; $P = 0.243$ and OR 1.03; 95% CI, 1–1.06; $P = 0.08$, respectively), as shown in **Table 2**.

Embryo Quality Parameters Association Analysis

Parent blood type combinations and embryo quality parameters, including fertilization rate, cleavage rate, and high-quality embryo rate were assessed by adjusting controlled ovarian hyperstimulation protocols. The combination of female blood type B and male blood type A has a negative association with fertilization rate (OR 0.98; 95% CI, 0.97–1; $P = 0.01$), while the combination of female blood type O and male blood type A has a slightly negative association with cleavage rate (OR 1; 95% CI, 0.99–1; $P = 0.027$). As evident in **Table 3**, no statistically significant difference was observed between the ABO blood group of mother/father mating blood types and High-Quality Embryo Rate.

Assisted Reproductive Technology (ART) Outcomes Association Analysis

Outcomes of treatment to blood groups, including Biochemical and clinical Pregnancy, early abortion rate, and live birth rate were further compared. As shown in **Table 4**, there was a statistically significant positive association between the combination of female blood type AB and male blood type AB with biochemical pregnancy, clinical pregnancy, and live birth rate (OR 1.36; 95% CI, 1.05–1.78; $P = 0.02$ and OR 1.31; 95% CI, 1.03–1.68; $P = 0.031$ and OR 1.28; 95% CI, 1.01–1.63; $P = 0.041$, respectively). No statistically significant difference was observed between couple combinations for ABO blood groups and early abortion rate.

Birth Sex, Weights, Defects Rate Association Analysis

Finally, a multivariate logistic regression analysis was performed to determine the relationship between blood type and birth sex,

TABLE 1 | Overall demographics and patient characteristics of the study population stratified by female blood type (n = 30,717).

Characteristics	All	A (n = 8,846)	B (n = 9,712)	AB (n = 3,257)	O (n = 8,902)	P-value
Age (y)*	30.06 ± 2.83	30.09 ± 2.84	30.06 ± 2.85	29.98 ± 2.82	30.05 ± 2.81	0.236
BMI (kg/m^2)*	22.83 ± 3.22	22.82 ± 3.20	22.83 ± 3.19	22.91 ± 3.36	22.82 ± 3.20	0.554
Duration of subfertility (m)*	48.22 ± 33.58	48.47 ± 33.31	48.36 ± 34.09	47.68 ± 32.06	48.03 ± 33.83	0.613
Primary subfertility (%)#	44.93% (13,801/30,717)	45.16% (3,995/8,846)	45.53% (4,422/9,712)	45.26% (1,474/3,257)	43.92% (3,910/8,902)	0.145
Infertility diagnoses (%)#						0.056
Anovulation	12.79% (3,930/30,717)	13.60% (1,203/8,846)	12.26% (1191/9,712)	13.57% (442/3,257)	12.29% (1,094/8,902)	
Tubal factor	42.25% (12,979/30,717)	42.47% (3,757/8,846)	41.85% (4,064/9,712)	41.17% (1341/3,257)	42.88% (3,817/8,902)	
Male factor	3.16% (972/30,717)	3.18% (281/8846)	3.22% (313/9712)	2.86% (93/3257)	3.20% (285/8,902)	
Unexplained	29.41% (9034/30717)	29.11% (2,575/8,846)	29.73% (2,887/9,712)	29.92% (968/3,257)	29.25% (2,604/8,902)	
Combined	12.38% (3,802/30,717)	11.64% (1030/8846)	12.94% (1,257/9,712)	12.68% (413/3,257)	12.34% (1,102/8,902)	
Basal FSH (IU/L)*	6.645 ± 2.32	6.923 ± 2.28	6.958 ± 2.22	6.827 ± 2.12	6.983 ± 2.52	0.085
Basal LH (IU/L)*	4.810 ± 3.53	4.840 ± 3.55	4.75 ± 3.54	4.82 ± 3.52	4.83 ± 3.50	0.708
Basal T (ng/mL)*	0.26 ± 0.25	0.26 ± 0.22	0.27 ± 0.29	0.26 ± 0.23	0.26 ± 0.25	0.060
Basal E2(pg/mL)*	25.86 ± 32.73	26.02 ± 31.53	26.02 ± 33.22	35.86 ± 32.97	25.47 ± 33.26	0.956
Basal P (ng/mL)*	0.30 ± 0.70	0.30 ± 0.66	0.30 ± 0.74	0.30 ± 0.68	0.30 ± 0.69	0.427
COH/FET Protocols (%)#						0.299
Short GnRH-a long protocol	24.80% (7,617/30,717)	24.34% (2,153/8,846)	24.80% (2,409/9,712)	24.07% (784/3,257)	25.51% (2,271/8,902)	
GnRH-a prolonged protocol	31.95% (9,813/30,717)	31.82% (2,815/8,846)	31.41% (3,051/9,712)	32.32% (1,056/3,257)	32.48% (2,891/8,902)	
Natural cycle	14.56% (4,472/30,717)	14.44% (1,277/8,846)	14.52% (1,410/9,712)	14.95% (487/3,257)	14.58% (1,298/8,902)	
Artificial cycle	28.70% (8,815/30,717)	29.40% (2,601/8,846)	29.26% (2,842/9,712)	28.55% (930/3,257)	27.43% (2,442/8,902)	
Dosage of rFSH (IU)*	2150 ± 649.72	2142 ± 657.90	2152 ± 653.65	2112.1 ± 644.34	2169 ± 639.14	0.049[a]
Peak E2 level on day of hCG trigger (pg/mL)*	3890.4 ± 2238.8	3885.4 ± 2232.3	3896.9 ± 2217.06	3930.4 ± 2257.78	3874.0 ± 2261.55	0.956
Peak endometrial thickness (mm)*	12.20 ± 2.42	12.24 ± 2.41	12.15 ± 2.41	12.21 ± 2.44	12.21 ± 2.44	0.157
Number of retrieved oocytes (n)*	12.56 ± 5.66	12.60 ± 5.63	12.43 ± 5.66	12.84 ± 5.56	12.59 ± 5.77	0.063
Percentage of MII oocytes (%)	0.817 ± 0.16	0.818 ± 0.16	0.817 ± 0.16	0.819 ± 0.16	0.816 ± 0.16	0.159
Good-quality embryos (%)#	0.67 ± 0.27	0.672 ± 0.26	0.667 ± 0.26	0.675 ± 0.26	0.671 ± 0.27	0.587
The Number of ET (n)*	1.79 ± 0.51	1.80 ± 0.52	1.79 ± 0.51	1.78 ± 0.52	1.78 ± 0.51	0.211
Stage of embryos Transferred (%)#						0.415
Cleavage stage	73.46% (22,566/30,717)	73.78% (6,527/8,846)	73.03% (7,093/9,712)	72.80% (2,371/3,257)	73.86% (6,575/8,902)	
Blastocyte stage	26.54% (8,151/30,717)	26.22% (2,319/8,846)	26.97% (2,619/9,712)	27.20% (886/3,257)	26.14% (2,327/8,902)	
Percentages of fresh ET or FET (%)#						0.046[a]
Fresh ET	56.74% (17,430/30,717)	56.16% (4,968/8,846)	56.22% (5,460/9,712)	56.49% (1,840/3,257)	57.99% (5,162/8,902)	
FET	43.26% (13,287/30,717)	43.84% (3,878/8,846)	43.78% (4,252/9,712)	43.51% (1,417/3,257)	42.01% (3,740/8,902)	

**Indicates continuous variables, presented as mean ± standard deviation (SD). #Indicates categorical variables, presented as percentage (number).*

[a]Indicates $P < 0.05$, but no significant difference between each group. BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone; E2, estradiol; P, progesterone; COH, controlled ovarian hyperstimulation; FET, frozen-thawed embryo transfer; rFSH, recombinant follicle stimulating hormone; ET, embryo transfer.

TABLE 2 | Multivariate logistic regression analyses between the history of spontaneous miscarriage and parental blood type combinations.

Female blood type	Male blood type	Dosage model		Dominant model		Continuous model	
		OR (95%CI)	*P*-value	OR (95%CI)	*P*-value	OR (95%CI)	*P* value
AB	B	1.00 (0.99–1.01)	0.874	0.99 (0.97–1.01)	0.433	0.99 (0.96–1.02)	0.384
AB	A	1.00 (0.99–1.01)	0.753	0.99 (0.97–1.01)	0.348	1.00 (0.97–1.03)	0.843
AB	O	1.01 (1.00–1.01)	0.243	1.03 (1.00–1.05)	0.023	1.03 (1.00–1.06)	0.080
AB	AB	0.99 (0.98–1.01)	0.304	0.99 (0.96-1.02)	0.426	0.98 (0.94–1.02)	0.323
B	B	1.00 (0.99–1.00)	0.521	1.00 (0.99–1.01)	0.871	0.99 (0.97–1.01)	0.438
B	A	1.00 (0.99–1.01)	0.966	1.01 (0.99–1.02)	0.262	1.01 (0.99–1.03)	0.213
B	O	1.00 (1.00–1.01)	0.443	0.99 (0.98–1.01)	0.214	0.99 (0.98–1.01)	0.566
B	AB	1.00 (0.99–1.01)	0.963	1.00 (0.98–1.03)	0.646	1.00 (0.98–1.03)	0.802
A	B	1.00 (1.00–1.01)	0.465	1.01 (0.99–1.02)	0.433	1.02 (1.00–1.04)	0.113
A	A	1.00 (0.99–1.00)	0.467	0.99 (0.98–1.01)	0.355	0.99 (0.97–1.01)	0.166
A	O	1.00 (0.99–1.00)	0.525	1.00 (0.98–1.01)	0.561	0.99 (0.97–1.01)	0.388
A	AB	1.00 (1.00–1.01)	0.373	1.01 (0.99–1.03)	0.308	1.01 (0.98–1.04)	0.370
O	B	1.00 (0.99–1.01)	0.993	1.00 (0.99–1.01)	0.910	1.00 (0.98–1.02)	0.816
O	A	1.00 (1.00–1.01)	0.321	1.00 (0.99–1.02)	0.676	1.00 (0.98–1.02)	0.806
O	O	1.00 (0.99–1.00)	0.356	1.00 (0.99–1.02)	0.731	1.00 (0.98–1.02)	0.767
O	AB	1.00 (0.99–1.01)	0.885	0.99 (0.97–1.01)	0.348	0.99 (0.96–1.02)	0.635

TABLE 3 | Multivariate logistic regression analyses between embryo quality parameters and parental blood type combinations by adjusting for controlled ovarian hyperstimulation protocols.

Female blood type	Male blood type	Fertilization rate		Cleavage rate		High quality embryo rate	
		OR (95%CI)	*P*-value	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value
B	A	0.98 (0.97–1.00)	0.010	1.00 (1.00–1.01)	0.251	0.99 (0.97–1.01)	0.354
B	O	1.01 (0.99–1.02)	0.245	1.00 (1.00–1.00)	0.942	1.02 (1.00–1.04)	0.110
B	B	1.01 (0.99–1.02)	0.315	1.00 (1.00–1.00)	0.783	0.99 (0.97–1.01)	0.442
B	AB	1.01 (0.98–1.03)	0.563	1.00 (0.99–1.00)	0.254	1.00 (0.97–1.03)	0.843
AB	A	1.00 (0.98–1.02)	0.813	1.00 (1.00–1.01)	0.343	0.99 (0.96–1.02)	0.564
AB	O	1.00 (0.98–1.03)	0.854	1.00 (0.99–1.00)	0.386	1.01 (0.98–1.04)	0.520
AB	B	1.00 (0.98–1.02)	0.847	1.00 (0.99–1.01)	0.818	1.01 (0.98–1.04)	0.529
AB	AB	1.01(0.97–1.04)	0.701	1.00 (0.99–1.01)	0.832	0.98 (0.94–1.02)	0.313
A	A	1.01 (0.99–1.02)	0.278	1.00 (1.00–1.01)	0.654	1.01 (0.99–1.03)	0.510
A	O	1.00 (0.99–1.02)	0.839	1.00 (0.99–1.00)	0.464	0.98 (0.96–1.00)	0.061
A	B	0.99 (0.98–1.01)	0.282	1.00 (1.00–1.00)	0.992	1.01 (0.99–1.03)	0.308
A	AB	1.00 (0.97–1.02)	0.782	1.00 (1.00–1.01)	0.658	1.00 (0.97–1.03)	0.816
O	A	1.01 (1.00–1.03)	0.089	1.00 (0.99–1.00)	0.027	1.01 (0.99–1.03)	0.497
O	O	0.99 (0.97–1.00)	0.127	1.00 (1.00–1.01)	0.171	1.00 (0.98–1.02)	0.860
O	B	1.00 (0.99–1.02)	0.840	1.00 (1.00–1.00)	0.680	0.99 (0.98–1.01)	0.508
O	AB	0.99 (0.97–1.02)	0.565	1.00 (1.00–1.01)	0.559	1.00 (0.98–1.03)	0.808

birth weights, and birth defects rate in single live born cycles. As shown in **Table 5**, no blood type was statistically significantly associated with birth sex, birth weight, and birth defect rate.

DISCUSSION

To the best of our knowledge, this study represents the largest retrospective cohort study evaluating the association between parent ABO blood group pairs and ART outcomes published so far. The result suggests there was a statistically significant positive association between the combination of female blood type AB and male blood type AB with biochemical pregnancy, clinical pregnancy, and live birth rate. No statistically significant difference was observed between couple combinations for ABO blood groups and High-quality embryo rate, early abortion rate, birth sex, birth weights, and birth defect rate.

As early as 1943, Levine identified ABO incompatibility as a cause of early abortions and stillbirths. By analyzing the relation between mother/father joint ABO blood group in 79 couples suffering from recurrent abortion in India, Malekasgar

TABLE 4 | Multivariate logistic regression analyses between assisted reproductive technology (ART) outcomes and parental blood type combinations with adjustment for the number of transfer embryo(s).

Female blood type	Male blood type	Biochemical pregnancy		Clinical pregnancy		Early abortion rate		Live birth rate	
		OR (95%CI)	*P*-value	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value
AB	B	1.04 (0.88–1.23)	0.614	0.93 (0.79–1.09)	0.374	0.87 (0.69–1.10)	0.255	0.91 (0.78–1.06)	0.234
AB	A	0.92 (0.78–1.10)	0.361	0.98 (0.83–1.16)	0.824	1.08 (0.87–1.35)	0.479	0.96 (0.82–1.13)	0.646
AB	O	0.90 (0.76–1.07)	0.239	0.97 (0.83–1.14)	0.742	1.04 (0.83–1.30)	0.708	1.02 (0.87–1.20)	0.769
AB	AB	1.36 (1.05–1.78)	0.020	1.31 (1.03–1.68)	0.031	1.01 (0.71–1.42)	0.975	1.28 (1.01–1.63)	0.041
B	B	0.99(0.89–1.10)	0.841	0.98 (0.88–1.09)	0.749	1.19 (0.94–1.50)	0.155	0.99 (0.90–1.10)	0.920
B	A	0.97 (0.86–1.09)	0.585	1.00 (0.90–1.11)	0.972	0.83 (0.66–1.05)	0.119	0.99 (0.89–1.10)	0.809
B	O	1.06 (0.95–1.19)	0.305	1.05 (0.94–1.17)	0.383	0.96 (0.76–1.21)	0.738	1.07 (0.96–1.19)	0.222
B	AB	0.97(0.82–1.14)	0.678	0.94 (0.80–1.10)	0.458	1.17 (0.81–1.65)	0.395	0.90 (0.77–1.05)	0.196
A	B	0.99 (0.88–1.11)	0.861	1.03 (0.93–1.15)	0.572	1.01 (0.71–1.42)	0.959	1.01 (0.91–1.13)	0.802
A	A	1.05 (0.93–1.18)	0.435	0.99 (0.88–1.10)	0.806	1.13 (0.81–1.56)	0.477	1.03 (0.93–1.15)	0.556
A	O	0.97 (0.86–1.08)	0.550	0.96 (0.86–1.08)	0.515	0.82 (0.58–1.16)	0.275	0.92 (0.83–1.03)	0.137
A	AB	1.00 (0.84–1.19)	0.981	1.04 (0.88–1.23)	0.623	1.15 (0.68–1.86)	0.593	1.09 (0.92–1.28)	0.315
O	B	1.00 (0.90–1.12)	0.962	1.02 (0.92–1.14)	0.690	0.97 (0.76–1.23)	0.781	1.04 (0.93–1.15)	0.500
O	A	1.03 (0.91–1.15)	0.680	1.02 (0.92–1.15)	0.675	1.04 (0.83–1.31)	0.714	1.00 (0.89–1.11)	0.971
O	O	1.02 (0.91–1.14)	0.739	1.00 (0.89–1.11)	0.966	1.08 (0.86–1.37)	0.490	1.00 (0.9–1.120)	0.980
O	AB	0.90 (0.77–1.07)	0.242	0.91 (0.77–1.07)	0.240	0.78 (0.53–1.13)	0.201	0.92 (0.79–1.08)	0.303

TABLE 5 | Multivariate logistic regression analyses between birth sex, birth weights, birth defects, and parental blood type combinations in single live born cycles.

Female blood type	Male blood type	Birth sex		Birth weights		Birth defects	
		OR (95%CI)	*P*-value	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value
AB	B	0.94 (0.72–1.22)	0.630	1.00 (0.98–1.02)	0.936	0.80 (0.34–1.75)	0.598
AB	A	1.02 (0.78–1.34)	0.864	1.00 (0.98–1.02)	0.832	1.64 (0.78–3.38)	0.182
AB	O	0.95 (0.73–1.24)	0.698	1.00 (0.98–1.02)	0.898	0.89 (0.39–1.90)	0.764
AB	AB	1.19 (0.82–1.73)	0.365	1.01 (0.98–1.04)	0.528	0.61 (0.14–1.90)	0.447
B	B	1.18 (0.99–1.41)	0.059	1.01 (0.99–1.03)	0.571	0.72 (0.37–1.32)	0.297
B	A	1.02 (0.85–1.22)	0.853	1.01 (0.99–1.03)	0.402	1.00 (0.55–1.80)	0.994
B	O	0.85 (0.71–1.01)	0.064	0.99 (0.97–1.01)	0.319	1.55 (0.86–2.78)	0.143
B	AB	0.95 (0.74–1.23)	0.696	0.99 (0.96–1.02)	0.546	0.73 (0.26–1.79)	0.510
A	B	0.96 (0.80–1.14)	0.630	1.01 (0.98–1.04)	0.479	1.19 (0.65–2.14)	0.558
A	A	0.86 (0.72–1.04)	0.117	1.01 (0.98–1.04)	0.517	0.71 (0.38–1.29)	0.274
A	O	1.14 (0.95–1.37)	0.166	0.98 (0.95–1.01)	0.211	0.92 (0.50–1.68)	0.799
A	AB	1.14 (0.88–1.49)	0.322	1.00 (0.96–1.04)	0.872	1.63 (0.69–3.71)	0.250
O	B	0.90 (0.76–1.08)	0.264	0.99 (0.97–1.01)	0.336	1.26 (0.71–2.21)	0.419
O	A	1.12 (0.94–1.35)	0.209	0.99 (0.97–1.01)	0.306	1.01 (0.57–1.77)	0.967
O	O	1.08 (0.90–1.30)	0.409	1.02 (1.00–1.04)	0.054	0.76 (0.41–1.36)	0.363
O	AB	0.84 (0.65–1.10)	0.203	1.00 (0.97–1.03)	0.918	1.04 (0.42–2.36)	0.935

et al. show an excess of joint "A/B" blood groups in couples with RSA (17). However, this study presented the difference simply by the percentage of blood types not the *P*-value of statistical analysis. By screening the ABO blood groups in aborted fetuses and their parents in 124 early spontaneous abortions, Bandyopadhyay found a significantly higher ($P < 0.05$) frequency of ABO incompatibility in couples with miscarriage when compared with newborns and their parents from the same area (18). This conclusion indicates that the ABO incompatibility between the father and mother may be a risk factor for early spontaneous abortions. However, these results lacked statistical power to detect associations of blood groups with history of spontaneous abortions before ART treatment, or early in spontaneous abortions following IVF-ET.

Awartani et al. (7) compared the clinical parameters of 566 IVF treatment cycles with different ABO blood types and found no significant association between blood type and pregnancy rate. This result agrees with another retrospective study (19) that evaluated the effect of non-O blood type on clinical pregnancy of 497 women and found no statistically significant association between them. For live birth, two retrospective studies on infertile women undergoing IVF came to different conclusions. One study in a cohort of 626 infertile women suggested that women with blood type B had a significantly higher likelihood of live birth following IVF-ET (9). However, Pereira et al. observed no relation of blood groups to live-birth rate by assessing 2,329 patients undergoing fresh IVF with day 5 single embryo transfer (10). In our study population, couples who have joint "AB/AB" blood types had a significantly higher likelihood of biochemical pregnancy, clinical pregnancy, and live birth. Using the ABO incompatibility effect infertility hypothesize is easy to explain our findings, considering that the same blood types were completely compatible and no antibodies were activated to attack each other.

No association between ABO blood type and birth weight at delivery was found in our study, which reflects the conclusions of Pereira (10). In 1975, Allan reported that the birth sex ratio of male to female babies is related to the ABO blood group of babies and mothers by studying 53,679 mother-baby combinations. AB mothers were found to have higher sex ratio babies while A babies have a lower sex ratio (20). The authors suggest that these differences may be caused by the interaction of the ABO genes and some unknown sex-determining genes with estrogen and progesterone. No correlation was found between blood type and birth sex in our cohort.

As mentioned before, the current evidence available is not sufficient to confirm that the blood type ABO is related to some aspects of female fertility. The underlying mechanisms of ABO blood type incompatibility may play some role in abortion. Some authors hypothesized that ABO blood type may result in infertility because of the presence of incompatible antibodies of spermatozoa in the serum or the secretions of the mother's genital tract. Genetics is another possible mechanism, given the candidate *NR5A1* and *TGFBR1* genes impacting oocyte quality or early implantation are in proximity to the 9q34 locus of the ABO gene (21). The other possible mechanism of ABO-related infertility was its effect on thrombosis, some studies suggested the formation of blood vessels at the maternal-fetal interface may lead to the failure of implantation or placenta, further influent the clinical outcomes of IVF (22). The relationship between non-O blood type and venous thromboembolism has been observed in previous studies (23) but data remains controversial (24). In consideration of the maternal-fetal differences in ABO membrane protein structure, studies on couple combinations for ABO blood groups were not sufficient to address the underlying mechanisms.

This study design has a number of limitations (observational, non-randomized, and retrospective). AMH level is missing for patients collected between January 2010 and December 2019. Most of them did not receive serum AMH testing, as this clinical test has only been used in our hospital since 2017. It is noteworthy that newborn blood type information is missing, and our results of no association between blood type and birth ratio do not exclude other possibilities. The mechanisms that could explain joint "AB/AB" blood types having higher success rates are not entirely clear. Thus, further prospective and mechanism studies are needed to prove our results. Nonetheless, our findings relating mother/father joint ABO blood group to clinical outcomes of IVF/ICSI, indicate the success rate of IVF/ICSI cycles in parent mating AB blood type is higher than that in other blood type combination groups. Our results provide evidence of the relationship between joint ABO blood group and IVF/ICSI clinical outcomes in a Chinese population.

CONCLUSION

In conclusion, the current large sample size retrospective cohort study demonstrated the statistically significant positive association between the combination of female blood type AB and male blood type AB with biochemical pregnancy, clinical pregnancy, and live birth rate. Couples who have joint "AB/AB" blood types had a significantly higher likelihood of success rate for IVF/ICSI cycles.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XB and YS designed the study. XB, FZ, and HS analyzed the data. XB, ZB, and YL wrote the manuscript. YS revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.813781/full#supplementary-material

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Preliminary prediction of semen quality based on modifiable lifestyle factors by using the XGBoost algorithm

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Introduction: Semen quality has decreased gradually in recent years, and lifestyle changes are among the primary causes for this issue. Thus far, the specific lifestyle factors affecting semen quality remain to be elucidated.

Materials and methods: In this study, data on the following factors were collected from 5,109 men examined at our reproductive medicine center: 10 lifestyle factors that potentially affect semen quality (smoking status, alcohol consumption, staying up late, sleeplessness, consumption of pungent food, intensity of sports activity, sedentary lifestyle, working in hot conditions, sauna use in the last 3 months, and exposure to radioactivity); general factors including age, abstinence period, and season of semen examination; and comprehensive semen parameters [semen volume, sperm concentration, progressive and total sperm motility, sperm morphology, and DNA fragmentation index (DFI)]. Then, machine learning with the XGBoost algorithm was applied to establish a primary prediction model by using the collected data. Furthermore, the accuracy of the model was verified *via* multiple logistic regression following *k*-fold cross-validation analyses.

Results: The results indicated that for semen volume, sperm concentration, progressive and total sperm motility, and DFI, the area under the curve (AUC) values ranged from 0.648 to 0.697, while the AUC for sperm morphology was only 0.506. Among the 13 factors, smoking status was the major factor affecting semen volume, sperm concentration, and progressive and total sperm motility. Age was the most important factor affecting DFI. Logistic combined with cross-validation analysis revealed similar results. Furthermore, it showed that heavy smoking (>20 cigarettes/day) had an overall negative effect on semen volume and sperm concentration and progressive and total sperm motility (OR = 4.69, 6.97, 11.16, and 10.35, respectively), while age of >35 years was associated with increased DFI (OR = 5.47).

Conclusion: The preliminary lifestyle-based model developed for semen quality prediction by using the XGBoost algorithm showed potential for clinical application and further optimization with larger training datasets.

KEYWORDS

lifestyles, semen quality, artificial intelligence, machine learning, extreme gradient boosting (XGBoost)

Introduction

Semen quality is an important determinant of male fertility (1, 2). In recent years, the semen quality has decreased, and this adverse trend has aroused widespread concern (3, 4). Many factors have been reported to affect semen quality, including demographic characteristics such as age and body mass; diseases such as endocrine or genetic problems, prostate disorders, seminal tract obstruction, and oncological diseases; environmental factors such as temperature changes, pollution, and electromagnetic radiation; and lifestyle factors such as smoking, alcohol intake, and staying up late (1, 5–10). Extensive research has indicated that unhealthy lifestyles are among the most important factors accounting for male reproductive disorders and decreased semen quality (6, 11). However, the specific lifestyle factors affecting semen quality remain to be elucidated. Furthermore, undertaking the relevant research required for this purpose is difficult because of lifestyle complexity (characterized by factors such as frequent changes or the involvement of various characteristics and confounding variables).

Machine learning, a branch of artificial intelligence (AI), is suitable for dealing with flexible relationships among predictor variables and outcomes in large datasets (12). The application of machine learning in multiple fields of medicine could help develop disease prediction models (13–15), and many studies have applied this approach to the analysis of semen parameters, such as morphology (16). However, there are few studies involving the application of AI in the prediction of the impact of lifestyles on semen quality. To our knowledge, thus far, only 2 small-sample ($n = 100$) studies have revealed the effects of lifestyle variations on semen parameters (17, 18). Furthermore, the volunteers recruited in these studies were young (age between 18 and 36 years) and the semen quality parameters included were limited or ambiguous. Therefore, further comprehensive and extensive research is warranted. Hence, in this study, XGBoost, a decision-tree based machine learning algorithm, was applied to analyze the association between the semen quality characteristics and the lifestyles associated by using data collected from 5,109 men examined in our reproductive medical center, so as to develop a preliminary model for semen quality prediction. Furthermore, the accuracy of the model was verified *via* multiple logistic regression analyses to determine the value of further study.

Materials and methods

Study design

This study was approved by the Ethics Committee of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University (No. 2019-185), and all participants recruited signed informed consent forms. As shown in the patient recruitment flowchart (Figure 1), from October 2019 to September 2021, 6,951 men examined in our center were recruited. Participants with a BMI < 32 and without chromosome abnormalities were included. The exclusion criteria were as follows: prostatic inflammation and organic injury, seminal tract obstruction, cancer, hypospadias, low testosterone levels, varicocele, mumps, cryptorchidism, diabetes, microdeletion of the Y chromosome azoospermia factor (AZF), hyperlipidemia, hypertension, and sexually transmitted diseases. Ultimately, 6,388 men were included after dropping 563 patients who met the exclusion criteria. According to the different evaluations intended, the routine seminal assay including semen volume, sperm concentration, and sperm motility, was performed for all participants, while sperm morphology tests and DNA fragmentation index (DFI) examination was performed in 3,018 and 2,209 participants, respectively. Each participant completed a baseline questionnaire before the semen analysis, and cases with missing questionnaire responses were excluded. Thus, the final dataset included 5,109 men whose semen volume, sperm concentration, and sperm motility were analyzed. Furthermore, sperm morphology and DFI analyses were performed in 2,511 and 1,812 participants, respectively.

Questionnaire variables

The questionnaire comprised 13 items including 10 pertaining to habitual lifestyles and three general conditions including age, abstinence period, and date of questionnaire completion; the details are listed in

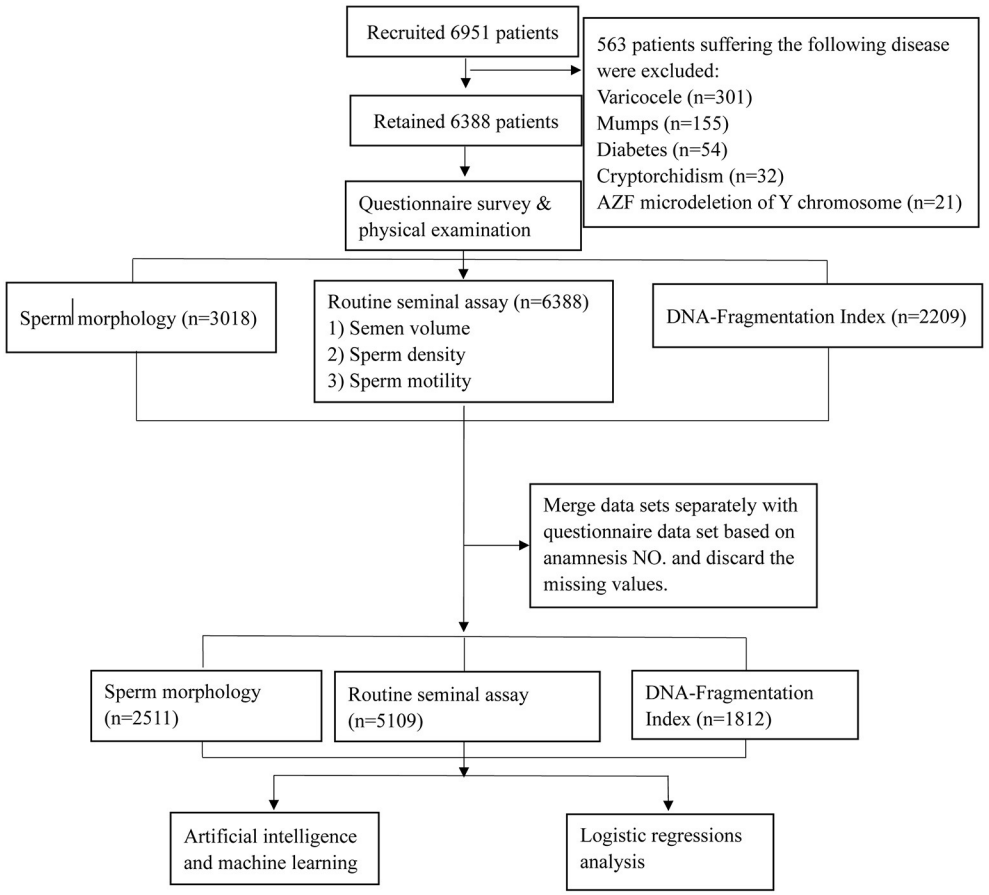

FIGURE 1
Flow chart of the study population. A total of 5,109 males were included in this study; all of these participants underwent a routine seminal assay while some also underwent sperm morphology and DNA Fragmentation Index assay according to the different inspection purposes.

Supplementary Table 1. Sleeplessness and intensity of sports activity were analyzed using the Insomnia Severity Index (Supplementary Table 2) and modified Physical Activity Questionnaire (Supplementary Table 3) (19), respectively.

Assessment of semen quality

Semen samples were collected in sterile plastic container by asking the participants to masturbate. The participants were asked to void urine and wash their hands and external genitalia before masturbating to provide the sample. The sample collected was placed in a water bath maintained at 37°C for 30–60 min for liquefaction. Semen volume was measured by weighing, assuming a semen density of 1.0 g/ml. Sperm concentration (spermatozoa N/mL) and motility (%) were evaluated using a computer-aided sperm analysis system. DFI was determined by flow cytometry after staining with acridine orange, and sperm morphology was investigated using the Diff-Quick staining method. Reference values from the World Health Organization semen analysis manual were used to assess semen characteristics (20), and values below the lower threshold provided in the WHO manual were defined as abnormal. Besides, the threshold of DFI 30% was applied to classify normal (DFI $<$ 30%) or abnormal (DFI $\geq$ 30%) groups according to a previously published article (21).

AI and machine learning

The algorithm used in this study was extreme gradient boosting (XGBoost). The feature importance was calculated by the gain method from the XGBoost python library, which worked by averaging training loss reduction caused by feature utilization for each splitting. The input variables were the information collected from the questionnaire

of each patient, and the output variables were the semen quality parameters. The input variables were considered categorical variables (Supplementary Table 1), and the output variables were considered dichotomous variables according to the criterion described above. The six semen quality parameters were independent indicators; the XGBoost model was developed using different hyperparameters, separately, to improve the accuracy of the algorithm.

Cross-validation was performed to adjust the parameters. First, a relatively high "learning_rate" was used and the optimum "n_estimators" was selected for this "learning_rate". Secondly, the parameters "max_depth" and "min_child_weight" were adjusted for the selected "learning_rate" and "n_estimators." Owing to the unbalanced category of the dataset, the training dataset was oversampled, and the "scale_pos_weight" was always equal to 1. Then, the learning rate was reduced. Next, "max_depth" was adjusted to simplify the XGBoost model according to the results obtained for the test dataset. The clean dataset used for XGBoost was randomly split into training and test datasets in a ratio of 70:30. Hyperparameter details are described in Table 1. Lastly, k-fold cross-validation with $k = 10$ was performed to evaluate machine learning models.

Statistical analysis and logistic regression analysis

Descriptive statistics were used to summarize general demographics. The correlations among 13 questionnaire items were evaluated by Pearson's correlation coefficients. For continuous variables, data are expressed as mean ± SD for normally distributed data or median (Interquartile range, IQR) values for non-parametric data. For categorical variables, data are expressed as percentages.

Univariate and multivariable logistic regression was used to identify the factors related to semen quality. For each independent variable, odds ratios (ORs) and 95% confidence intervals (CIs) were estimated. Collinearity analyses were performed before the logistic regression analysis, and the model's goodness-of-fit was graphically evaluated (ROC curves). The response variables were categorized per the method used for the XGBoost algorithm, and stepwise regression was applied for all multivariate logistic regression analyses. Moreover, k-fold cross-validation with $k = 10$ was performed to evaluate the accuracy of the model.

The univariate and multivariable logistic regression analyses were performed with SAS version 9.4 (SAS Institute, Cary, NC, USA), and k-fold cross-validation was performed with $k = 10$ by using the package for R (version 4.1.2). Other statistical analyses were performed using SPSS 23.0. $P < 0.05$ was considered statistically significant.

TABLE 1 Distribution of male participants whose data were used for machine learning and the hyperparameters for XGBoost.

Sperm quality parameters	Total no.	Train set no.	Test set no.	Learning rate	*N* estimators	Max depth	Min_child_weight	Scale_pos_weight
Semen volume	5,109	3,576	1,533	0.01	600	3	1	1
Sperm concentration	5,109	3,576	1,533	0.01	750	3	1	1
Progressive motility	5,109	3,576	1,533	0.01	600	3	1	1
Total motility	5,109	3,576	1,533	0.01	600	5	1	1
Sperm morphology	2,511	1,758	754	0.01	300	4	1	1
DFI	1,812	1,268	544	0.01	300	4	1	1

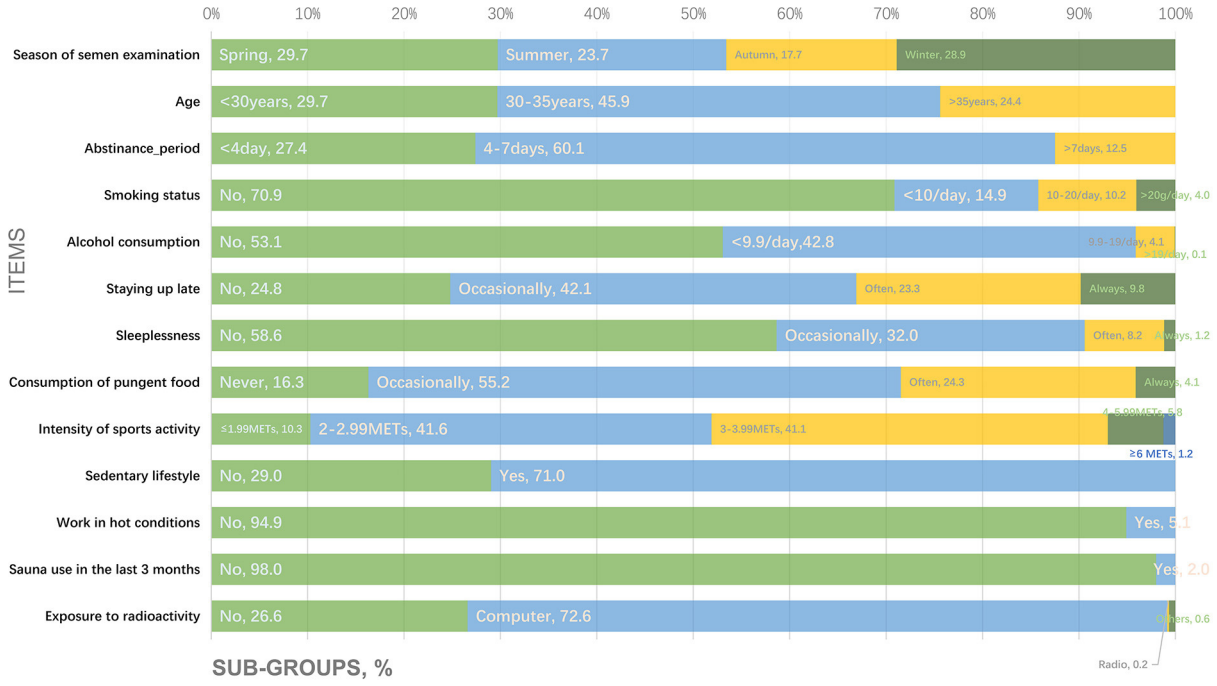

FIGURE 2
Information regarding the general and lifestyle characteristics of study participants.

Results

General information collected using the questionnaire

Figure 2 shows the proportions of participants corresponding to the subgroups for the following questionnaire items: (1) season of semen examination; (2) age; (3) abstinence period; (4) smoking status; (5) alcohol consumption; (6) staying up late; (7) sleeplessness; (8) consumption of pungent food; (9) intensity of sports activity; (10) sedentary lifestyle; (11) work in hot conditions; (12) sauna use in the last 3 months; and (13) exposure to radioactivity.

Semen quality among study participants

Among the 5,109 males, the median semen volume, sperm concentration, total sperm count, rapid progressive motility of the sperm, progressive motility of the sperm, and total sperm motility were 3.3 ml (95% CI: 3.40–3.49 ml), 68.1 $\times$ 10^6/ml (95% CI: 79.26–82.84 $\times$ 10^6/ml), 214.5 $\times$ 10^6 (95% CI: 262.28–275.23 $\times$ 10^6), 23.0% (95% CI: 22.76–23.50%), 47.8% (95% CI: 45.50–46.74%), and 60.4% (95% CI: 56.46–57.85%), respectively. The median normal sperm morphology among 2,511 men was 6.0% (95% CI: 6.24–6.51%), and the median DFI of 1,915 men was 14.4% (95% CI: 17.29–18.39%). In addition, 18.2% of the participants showed abnormal sperm morphology (morphologically normal forms, <4.0%, n = 2,511) and 13.9% had high DFI (≥30%, n = 1,812).

Risk factors affecting semen volume

We trained XGBoost with the input of the 13 items and achieved 60.7–70.3% accuracy, 55.4–72.5% sensitivity, and 39.9–70.4% specificity for the test set (Table 2).

The AUC of the XGBoost model for semen volume was 0.648 (Figure 3A) and the following cross-validation showed that the AUC of the model was 0.617. The feature importance plotted *via* XGBoost showed that the maximum score was for smoking status followed by abstinence period and staying up late (Figure 3B). Logistic regression analyses (Figure 3C) revealed that smoking status, abstinence period, sedentary lifestyle, and age were predictive markers of semen volume. The AUC of the combined markers (AUC = 0.655) was higher than that of the individual markers (AUC = 0.465, 0.563, 0.523, and 0.457, respectively), and the following cross-validation based on the multivariate regression analysis showed that the AUC of the model was 0.539. The maximum odds ratio was related to smoking status (OR = 4.69), indicating it to be the most important predictor (Supplementary Table 4).

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TABLE 2 Outcomes of machine learning using XGboost.

Sperm quality parameters	Classification accuracy	True negative	False positive	False negative	True positive	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Semen volume	0.7025	63	71	385	1,014	0.7248	0.4701	0.9346	0.1406
Sperm concentration	0.6758	94	87	410	942	0.6967	0.5193	0.9155	0.1865
Progressive motility	0.6282	269	147	423	694	0.6213	0.6466	0.8252	0.3887
Total motility	0.6067	218	157	446	712	0.6149	0.5813	0.8193	0.3283
DFI	0.6838	331	139	33	41	0.5541	0.7043	0.2278	0.9093
Sperm morphology	0.6167	55	83	206	410	0.6656	0.3986	0.8316	0.2107

Abstinence period, the second most important factor as revealed by XGBoost, was significantly associated with semen volume in the logistic regression analysis (Supplementary Table 4). Besides, as shown in Figure 3D, the OR per the regression analysis indicated that men who smoked more than 20 cigarettes/day were more likely to have a lower semen volume (OR: 4.69, 95% CI: 3.39–6.49, $P < 0.001$). However, males who smoked <10 cigarettes/day were less likely to have a lower semen volume (OR: 0.67, 95% CI: 0.48–0.93, $P < 0.05$) than non-smokers. Men who practiced abstinence for more than 7 days or had a sedentary lifestyle (≥5 h/day) were less likely to have a lower semen volume (OR: 0.63, 95% CI: 0.46–0.87, $P < 0.01$ and OR: 0.81, 95% CI: 0.65–1.00, $P < 0.05$, respectively).

Risk factors affecting sperm concentration

The AUC of the XGBoost model for sperm concentration was 0.661 (Figure 4A), and the cross-validation showed that the AUC of the model was 0.674. The feature importance plotted using XGBoost showed that the maximum important score was for smoking status, followed by age and season of semen examination (Figure 4B). The AUCs of the logistic regression analyses (Figure 4C) revealed that smoking status, age, intensity of sports activity, and consumption of pungent food were predictive markers of sperm concentration. The AUC of the combined marker (AUC = 0.680) was higher than those of individual markers (AUC = 0.457, 0.540, 0.519, and 0.489, respectively), and the cross-validation based on the multivariate regression analysis showed that the AUC of the model was 0.547. The maximum odds ratio was observed for smoking status (OR = 6.97), indicating it is the most important predictor (Supplementary Table 5). Age, the second-most important factor revealed by XGBoost, also showed significant association with sperm concentration *via* logistic regression assay (Supplementary Table 5). Besides, as shown in Figure 4D, males who smoked more than 20 cigarettes/day were more likely to have lower sperm concentrations than non-smokers (OR: 6.97, 95% CI: 5.18–9.37, $P < 0.001$), but smokers were less likely to have lower sperm concentrations than non-smokers when they smoked <10 cigarettes/day (OR: 0.13, 95% CI: 0.07–0.22, $P < 0.001$). Older men (>35 years) were less likely to have lower sperm density (OR: 0.72, 95% CI: 0.57–0.91, $P < 0.01$) than younger men (<30 years).

Risk factors affecting progressive sperm motility

The AUC of the XGBoost models for progressive sperm motility was 0.697 (Figure 5A), and the cross-validation showed

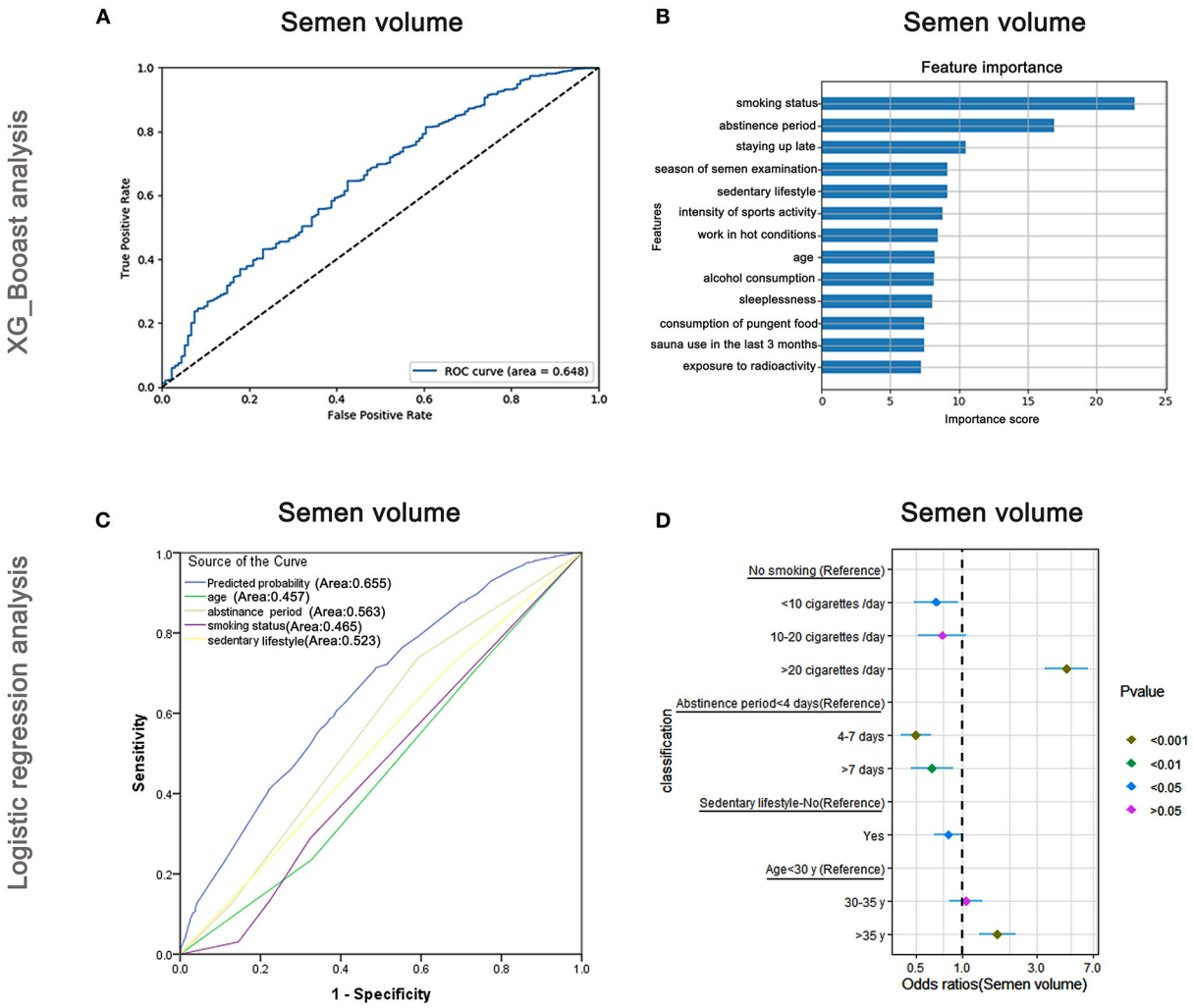

FIGURE 3
XGBoost and logistic regression analysis of the risk factors for semen volume. The ROC curve **(A)** and feature importance **(B)** analyzed by XGBoost and the ROC curve **(C)** and forest diagram showing significant risk factors **(D)** analyzed by logistic regression.

that the AUC of the model was 0.698. The feature importance plotted using XGBoost showed that smoking status was the most important factor, followed by abstinence period and alcohol consumption (Figure 5B). The AUCs of the logistic regression analyses (Figure 5C) revealed that smoking status, abstinence period, alcohol consumption, age, exposure to radioactivity, and working in hot conditions were predictive markers of progressive sperm vitality. The AUC of the combined marker (AUC = 0.705) was slightly higher than that of other markers, and the cross-validation based on the multivariate regression analysis showed that the AUC of the model was 0.696, which was similar to that of XGBoost (AUC = 0.697).

The top-two odds ratios were observed for smoking status and abstinence period (OR = 11.16 and 2.05), indicating their importance in predictions (Supplementary Table 6). Alcohol consumption, which was identified as the third-most important by XGBoost, also showed a significant association with progressive sperm motility in the logistic regression assay (Supplementary Table 6).

Moreover, as shown in Figure 5D and Supplementary Table 6, males who smoked more than 20 cigarettes/day were more likely to have lower progressive sperm motility (OR: 11.16, 95% CI: 7.82–15.93, $P < 0.001$) than non-smokers, but smokers were less likely to have lower progressive sperm motility than non-smokers when they smoked <10 cigarettes/day (OR: 0.07, 95% CI: 0.05–0.11, $P < 0.001$). Males who maintained abstinence for more than 7 days were more likely to show lower progressive sperm motility (OR: 2.05, 95% CI: 1.63–2.57, $P < 0.001$).

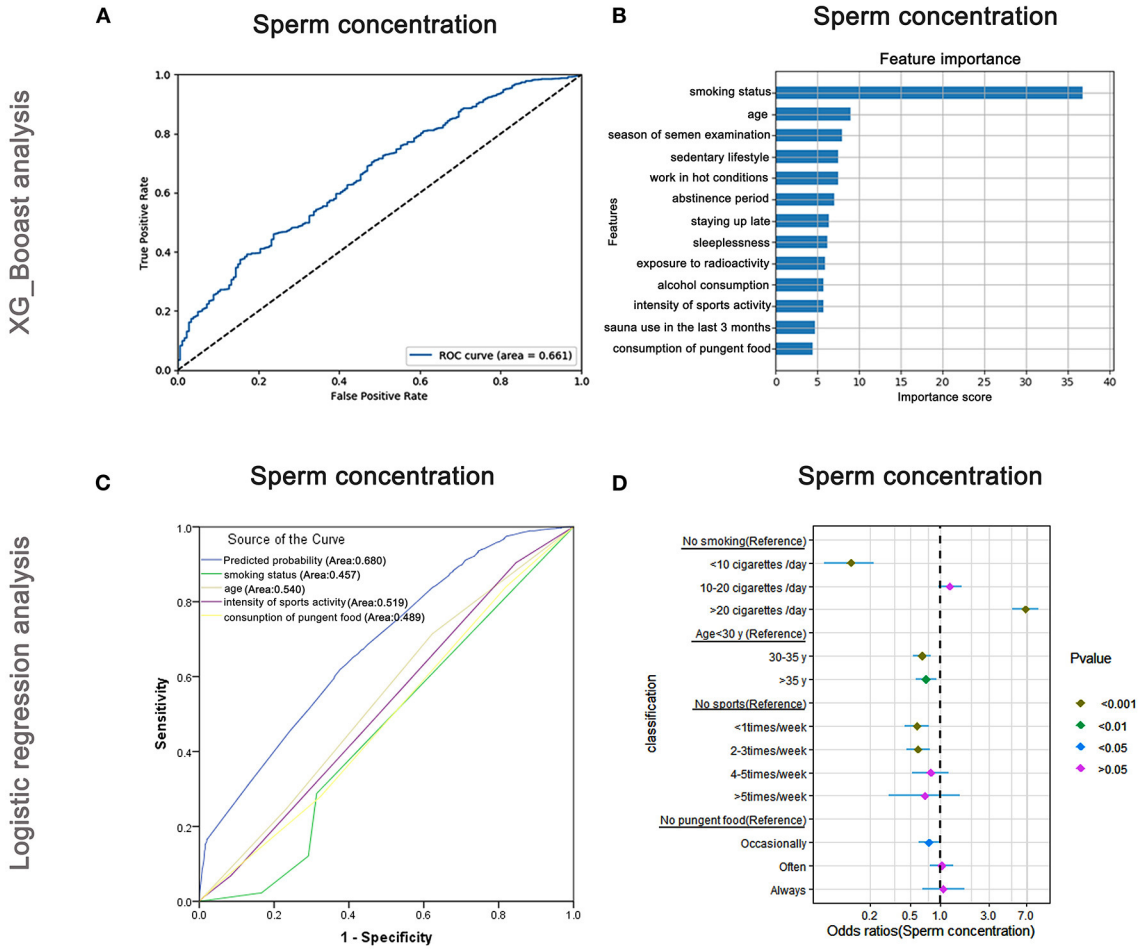

FIGURE 4
XGBoost and logistic regression analysis of the risk factors for sperm concentration. The ROC curve **(A)** and feature importance **(B)** analyzed by XGBoost and the ROC curve **(C)** and forest diagram showing significant risk factors **(D)** analyzed by logistic regression.

Risk factors affecting total sperm motility

The AUC of the XGBoost models for total sperm vitalities was 0.660 (Figure 6A), and the cross-validation showed that the AUC of the model was 0.686. The feature importance plotted *via* XGBoost showed that smoking status played the most important part, followed by working in hot conditions and abstinence period (Figure 6B). The AUCs of the logistic regression analyses (Figure 6C) revealed that smoking status, working in hot conditions, abstinence period, season of semen examination, alcohol consumption, consumption of pungent food, age, and exposure to radioactivity were predictive markers of total sperm vitality. The AUC of the combined marker (AUC = 0.700) was slightly higher than that of the other markers, and the cross-validation based on the multivariate regression analysis showed that the AUC of the model was 0.749. The maximum odds ratio was observed for smoking status (OR = 10.35), indicating it was the most important predictor (Supplementary Table 7). Moreover, working in hot conditions and abstinence period, two of the top-three important factors revealed by XGBoost, also significantly affected total sperm motility in the regression analysis (Supplementary Table 7).

As shown in Figure 6D and Supplementary Table 7, males who smoked more than 20 cigarettes/day were more likely to have lower total sperm motility than non-smokers (OR: 10.35, 95% CI: 7.35–14.56, $P < 0.001$), but smokers were less likely to have a lower total sperm motility than non-smokers when they smoked <10 cigarettes/day (OR: 0.06, 95% CI: 0.03–0.10, $P < 0.001$). Males who worked under hot conditions were less likely to show low total sperm motility (OR: 1.63, 95% CI: 1.20–2.21, $P < 0.05$). Moreover, males who maintained abstinence for more than 7 days were more likely to have lower total sperm motility (OR: 1.72, 95% CI: 1.37–2.17, $P < 0.001$).

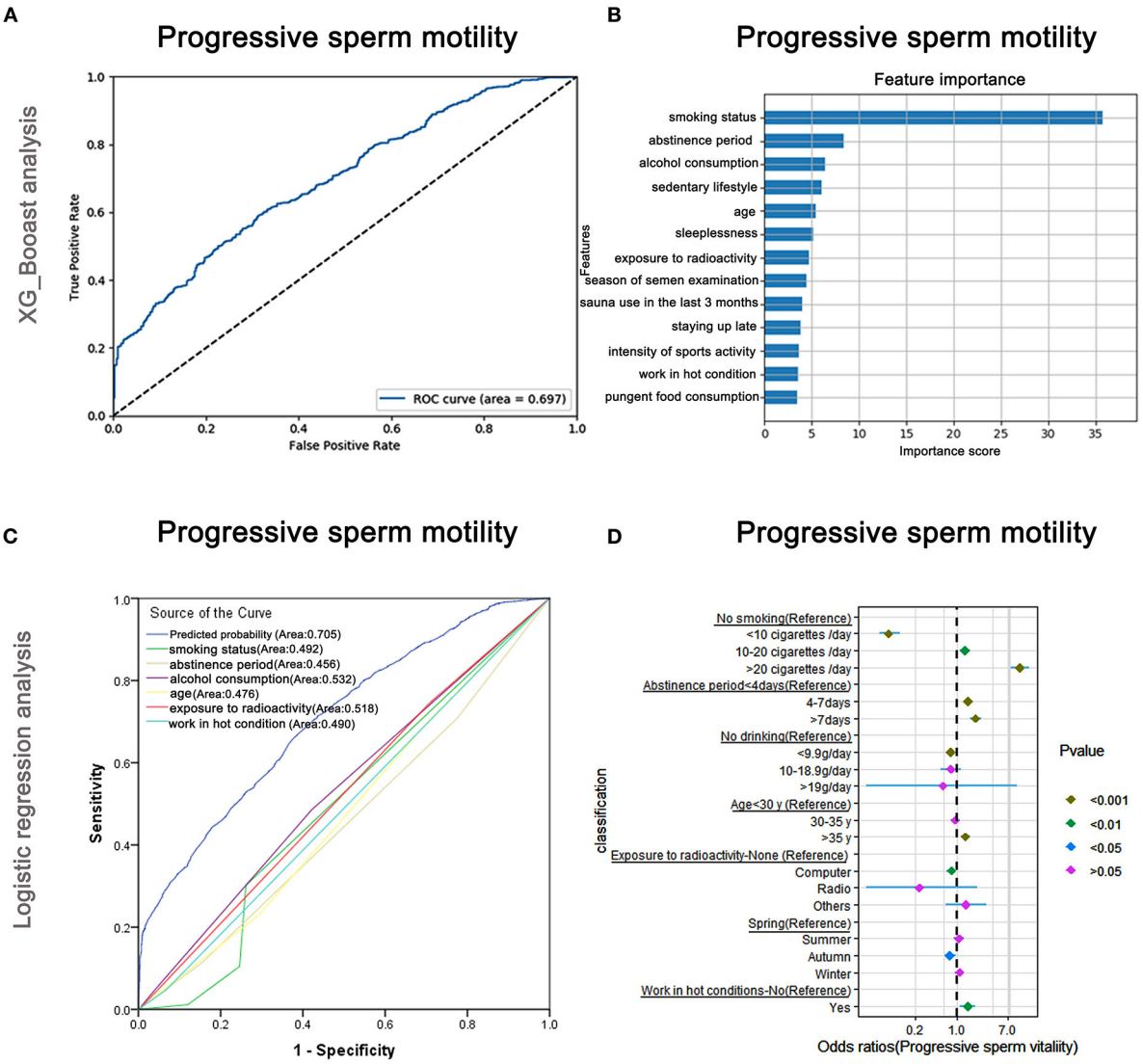

FIGURE 5
XGBoost and logistic regression analysis of the risk factors for progressive sperm motility. The ROC curve **(A)** and the feature importance **(B)** analyzed by XGBoost and the ROC curves **(C)** and forest diagram showing significant risk factors **(D)** analyzed by logistic regression.

Risk factors affecting sperm morphology

The AUC of the XGBoost model for sperm morphology was only 0.506 (Figure 7A), and the cross-validation showed that the AUC of the model was 0.520. The feature importance plot created using XGBoost showed that smoking status was the maximum important factor (Figure 7B). The AUCs of the logistic regression analyses (Figure 7C) revealed that smoking status was a predictive index for sperm morphology with a poor AUC (0.539), and the cross-validation based on the multivariate regression analysis showed that the AUC of the model was 0.543. As shown in Figure 7D and Supplementary Table 8, males who smoked more than 20 cigarettes/day were more likely to have abnormal sperm morphology than non-smokers (OR: 3.0, 95% CI: 1.76–5.12, $P < 0.001$), but the trend did not appear for males who smoked <10 cigarettes/day (OR: 0.53, 95% CI: 0.39–0.73, $P < 0.001$).

Risk factors affecting DFI

The AUC of the XGBoost model for DFI was 0.686 (Figure 8A), and the cross-validation showed that the AUC of the model was 0.697. The top three important features

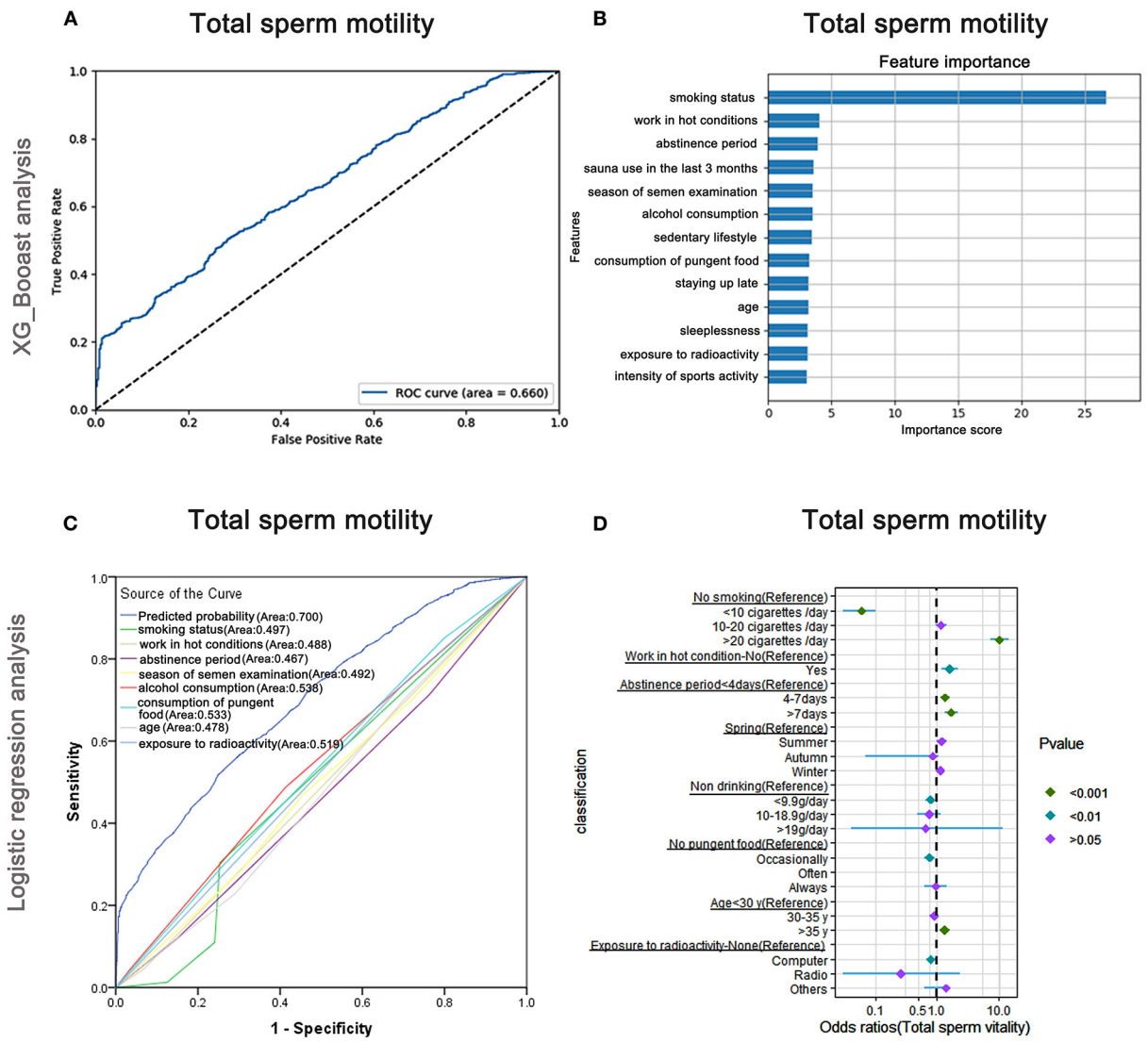

FIGURE 6
XGBoost and logistic regression analysis of the risk factors for total sperm motility. The ROC curve **(A)** and the feature importance **(B)** analyzed by XGBoost and the ROC curve **(C)** and forest diagram showing significant risk factors **(D)** analyzed by logistic regression.

affecting total sperm vitality were age, abstinence period, and smoking status (Figure 8B). The AUCs of the logistic regression analyses (Figure 8C) revealed that age, abstinence period, smoking status, and staying up late were predictive markers of sperm DFI. The AUC of the combined marker (AUC = 0.725) was higher than that of the other individual markers (AUC = 0.661, 0.598, 0.466, and 0.443). The cross-validation based on the multivariate regression analysis showed that the AUC of the model was 0.648. The top-two odds ratios appeared for age and abstinence period (OR = 5.47 and 3.61), indicating their important predictive roles. Smoking status, the third important factor revealed by XGBoost, was also shown to significantly affect sperm DFI in regression analysis (Supplementary Table 9).

Besides, as shown in Figure 8D and Supplementary Table 9, older males (>35 years) and those maintaining abstinence for more than 7 days were more likely to have higher DFI (OR: 5.47, 95% CI: 3.41–8.76, $P < 0.001$ and OR: 3.61, 95% CI: 2.27–5.75, $P < 0.001$) than younger males (<30 years old) and those maintaining abstinence for <4 days, respectively. Males who smoked <10 cigarettes /day were less likely to have a high DFI (OR: 0.42, 95% CI: 0.27–0.66, $P < 0.001$) than non-smokers. Nevertheless, when they smoked more than 20 cigarettes/day, the odds ratio of having high a DFI increased ($P > 0.05$).

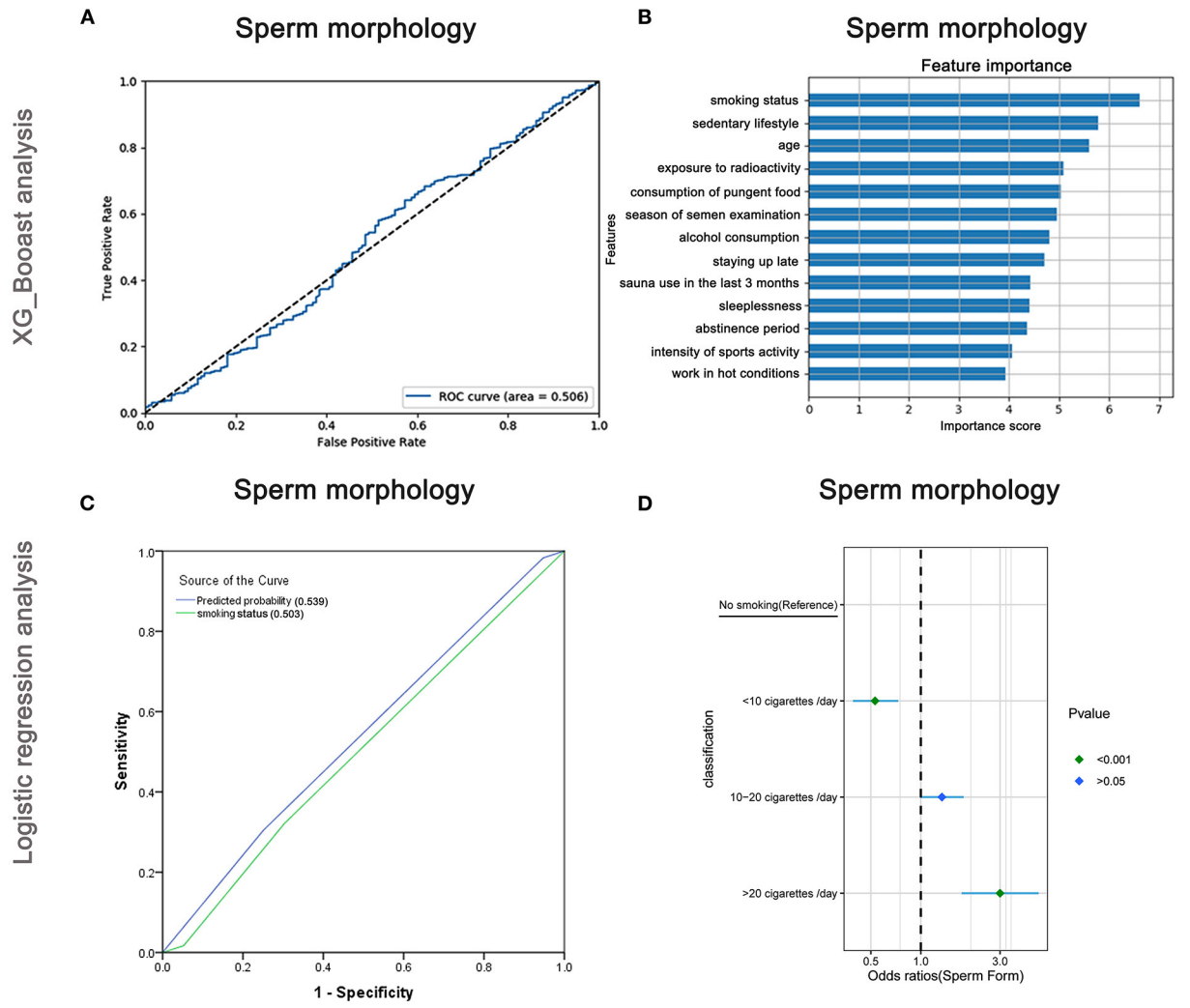

FIGURE 7
XGBoost and logistic regression analysis of the risk factors for sperm morphology. The ROC curve **(A)** and feature importance **(B)** analyzed by XGBoost and the ROC curve **(C)** and forest diagram showing significant risk factors **(D)** analyzed by logistic regression.

Correlations between general information

Considering the close relationships among the variables, Spearman rank correlation analysis was performed. As shown in Supplementary Table 10, significant positive correlations were observed between sedentary lifestyle and age, staying up late, sleeplessness, consumption of pungent food, and exposure to radioactivity, with correlation coefficient (ICC) values of 0.043, 0.078, 0.056, 0.061, and 0.438, respectively. Meanwhile, sedentary lifestyles showed negative correlations with smoking status, intensity of sports activity, and working in hot conditions (ICC = −0.088, −0.119, and −0.134, respectively). Positive correlations were observed between staying up late and smoking status, alcohol consumption, sleeplessness, consumption of pungent food, sedentary lifestyle, working in hot conditions, sauna use in the last 3 months, and exposure to radioactivity (ICC = 0.185, 0.238, 0.310, 0.342, 0.078, 0.087, 0.067, and 0.034; $P < 0.05$), but staying up late showed negative correlations with age, abstinence period, and intensity of sports activity (ICC = −0.074, −0.055, −0.067; $P < 0.05$). Exposure to radioactivity showed positive correlations with staying up late, consumption of pungent food, and a sedentary lifestyle (ICC = 0.034, 0.046, and 0.438; $P < 0.05$), but showed negative correlations with smoking status, alcohol consumption, and working in hot conditions (ICC = −0.142, −0.028, and −0.109).

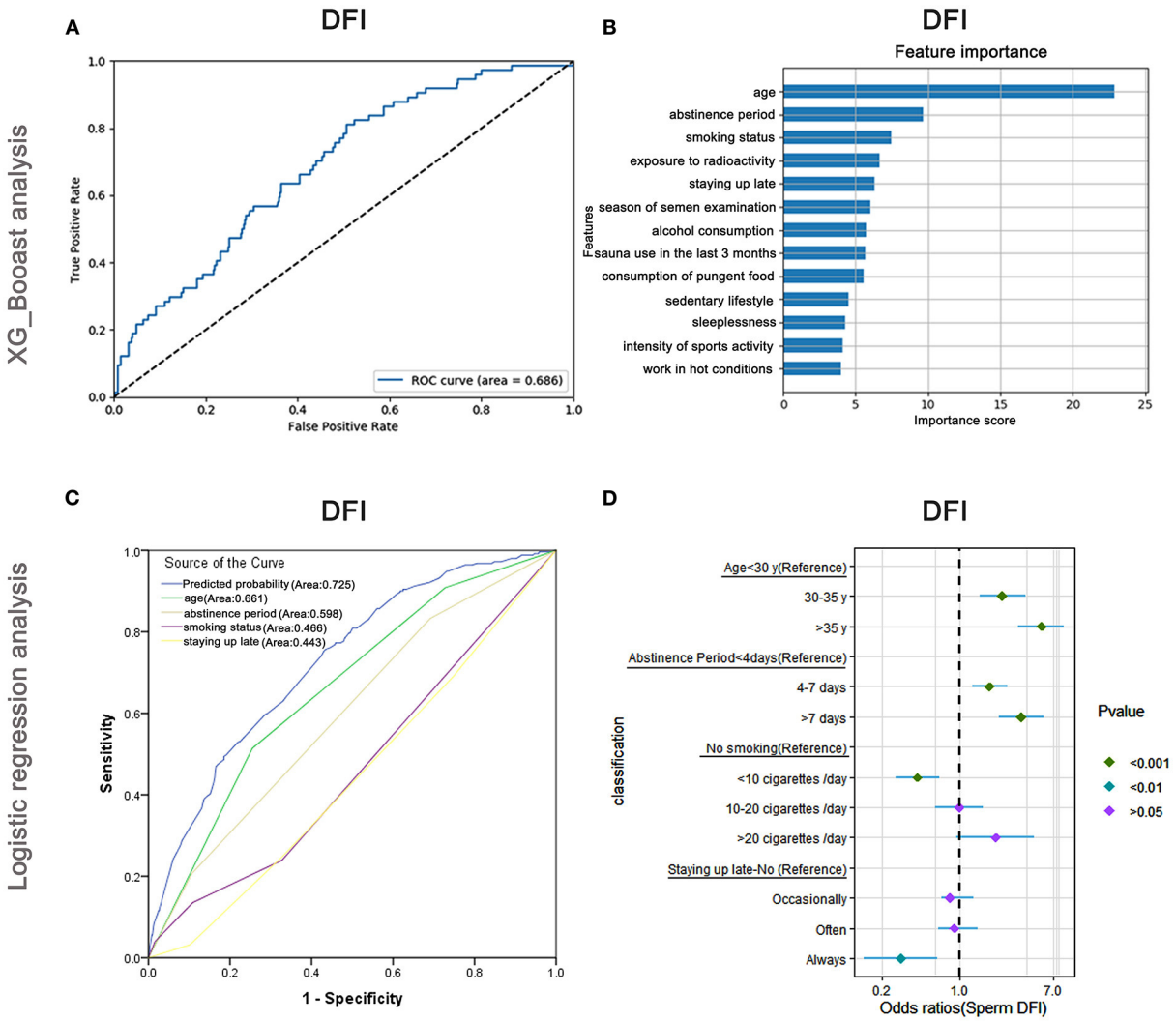

FIGURE 8
XGBoost and logistic regression analysis of the risk factors for the DNA fragmentation index (DFI). The ROC curve **(A)** and feature importance **(B)** analyzed by XGBoost and the ROC curve **(C)** and forest diagram showing significant risk factors **(D)** analyzed by logistic regression.

Discussion

The factors influencing semen quality are complex. Several studies have reported that male age and environmental/lifestyle exposures, rather than the genetic problems, are primarily responsible for abnormal semen quality (1, 22, 23). Among these, lifestyle factors can be easily modified without medical interventions (24), and elucidate the lifestyle factors affecting semen quality can guide men to take appropriate measures in the preconception period. However, as described above, the lifestyles leading to abnormal semen quality have not been completely clarified, while the complexity of these data made related analysis difficult. In recent years, the wide application of AI provided a new method for related research (13).

Since the typical tabular data in our research were more suitable for the decision tree algorithm, and XGBoost is generally superior to other decision tree algorithms such as GBDT random forest and artificial neural network models in terms of predictive performance (25–27), we constructed a preliminary lifestyle- and general factor-based semen quality prediction model *via* machine learning with the XGBoost algorithm by using data collected from 5,109 healthy men. Furthermore, since the accuracy of machine learning algorithms may be impaired because of overfitting or insufficient data training (12, 28–30), we have applied logistic regression combined with cross-validation to verify the accuracy and the feasibility of machine learning-based prediction model.

After training the XGBoost with 13 potential affecting factors, the results showed that the AUCs of semen volume, sperm concentration, sperm progressive and total sperm motility, and DFI were 0.648, 0.661, 0.697, 0.660, and 0.686, respectively, which was consistent with the regression model and the subsequent cross-validation. In addition, the top two important factors affecting semen volume, sperm concentration, and the top three important factors affecting sperm motility and DFI indicated by the XGBoost were also revealed as predictive indices by regression analysis, indicating the promising predictive value of machine learning. However, both the XGBoost model and logistic regression assay as well as the following cross-validation based on sperm morphology showed poor predictive values (AUC = 0.506, 0.520, 0.539, and 0.543). We speculate that this could be because lifestyle-related factors have minimal influence on sperm morphology (31), which is primarily mediated by genetic factors (32). The XGBoost prediction model indicated that smoking status was the most important factor affecting the parameters of semen volume, sperm concentration, and motility and was the third important factor affecting DFI, and the results were verified by regression analysis. Many other studies have also indicated cigarette smoking has an overall negative effect on the semen parameters because the toxins originating from cigarette smoke can decrease sperm mitochondrial activity and damage the chromatin structure in human sperm (33–36). The regression assay further revealed that heavy smoking (>20 cigarettes/day) posed a harmful effect, which suggested that men of reproductive age men should give up heavy smoking first. However, it was interesting that mild (<10 cigarettes/day) smoking had positive consequents, which was partly consistent with the findings of Kemal and Adelusi et al. (37, 38). They found that smokers showed a higher percentage of rapidly progressive sperm. The possible reason for this result is that mild smoking could generate trace amounts of oxides, which are required to support both sperm motility and capacitation (39). Moreover, our results inevitably showed interference since many patients who smoke very occasionally (<1 smoke/day) were categorized into the mild smoking group. Further adjustment and improvement of questionnaire designs will be performed in the following research.

Furthermore, the abstinence period was the second-most important factor influencing semen volume, progressive sperm motility, and DFI. The regression analysis further showed that longer abstinence periods (>7 days) can help increase semen volume, but would hurt sperm motility and increase sperm DFI. Sperm motility has been shown to peak within 4 or 5 days of abstinence (40), and spermatozoa accumulating in the epididymis might react with oxygen and nitrogen species (ROS and RNS) during prolonged periods of ejaculatory abstinence (41). Thus, males should maintain a healthy rhythm of sex to ensure optimal semen quality.

Age is the primary risk factor affecting semen DFI and a secondary risk factor affecting semen density. The regression assay revealed that the sperm DFI was higher in elder men, and oxidative stress damage might be one of the mechanisms underlying this finding (42–44). Meanwhile, the semen volume decreased and sperm density increased with increasing age, which might be attributable to prostate atrophy. Increased age is known to be associated with genome-wide mutations, DFI, and chromatin integrity (45), and high sperm DFI is associated with spontaneous abortion (46, 47). Thus, men should be to encouraged to have children early.

In addition, other factors explored in this study, except sauna use in the last 3 months and sleeplessness, influenced semen parameters to some extent. Curiously, unlike published research stating that a sedentary lifestyle or playing computer games adversely affected sperm motility (48), our regression analysis revealed that individuals with predominantly sedentary lifestyles were less likely to have lower semen volume and those exposed to computer radiation constantly were less likely to have lower sperm motility. Moreover, men who slept late were less likely to have a high DFI. However, the correlation analysis (please see the Supplementary Table 10) revealed that sedentary lifestyles and prolonged computer usage showed negative correlations with smoking status and late sleeping hours showed a negative correlation with age, which may be one reason for the confusing results.

Our study had some limitations. First, all data were collected from our own center without external validation, and we recruited patients receiving assisted fertility guidance or treatment, which may not fully represent the general population. Second, most lifestyle factors were self-reported and were subjective constructs in this research. Moreover, the stages of changes in most lifestyle factors could not be precisely delineated, and the valid data sample was not large enough to obtain precise predictions. Under the influence of the various factors described above, the current results showed that the XGBoost Algorithm had no obvious advantage over logistic regression. However, considering its benefits of allowing flexible analyses of relationships among predictor variables and outcomes in large datasets as well as the easy online updates in the prediction system, its implementation into the clinical workflow can be advantageous. We believe that the XGBoost will have promising predictive value and guiding significance after enlarging the data sample size and data feature dimensions as well adding information-based data extraction methods.

Conclusion

In summary, the preliminary model for predicting semen quality using lifestyle factors that was developed with the XGBoost algorithm had the potential to undergo further

optimization with larger training data. In addition, the model suggested that smoking status, abstinence period, and age were important factors affecting semen quality parameters.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

This study was approved by the Ethics Committee of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University (No. 2019-185), and all participants recruited signed informed consent forms.

Author contributions

BX, AZ, and YG designed the study and headed the interdisciplinary exchange. TY, YG, and HH performed machine learning. JL and MZ undertook the statistical analyses. WF examined semen parameters. MZ, BX, TY, and AZ collected the data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author TY is employed by Shanghai National Engineering Research Center of Digital Television Co., Ltd. This work is not funded by Shanghai National Engineering Research Center of Digital Television Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.811890/full#supplementary-material

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